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Prenatal Morphogenesis of Mammary Glands in Mouse and Rabbit

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Abstract Our understanding of prenatal morphogenesis of mammary glands has recently greatly advanced. This review focuses on morphogenesis proper, as well as cellular processes and tissue interactions involved in the progression of the embryonic mammary gland through sequential morphogenic stages in both the mouse and rabbit embryo. We provide a synthesis of both historical and more recent studies of embryonic mammary gland development, as well as arguments to revise old concepts about mechanisms of mammary line and rudiment formation. Finally, we highlight outstanding issues that remain to be addressed.

Keywords Mammary gland · Embryo · Rabbit · Mouse · Ectoderm · Epidermis · Appendage · Prenatal

Abbreviations

AR	Androgen Receptor
BrdU	5-bromo-2′-deoxyuridine
E	Embryonic day
FPP	Fat pad precursor
^{3}H-TdR	Tritiated desoxythymidine
ME	Mammary epithelium
ML	Mammary line
MM	Mammary mesenchyme
MR	Mammary rudiment

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Introduction

The mammary gland characterizes the Class of Mammals, to which it gives its name, meaning "of the breast" [1]. Its secreted milk feeds a mother's young offspring and provides immune support, while nursing also fosters a close relationship between both generations, which may benefit the young throughout their life. Therefore, mammary gland development, in particular its embryonic phase, has long been a topic of interest for zoologists. While many studies in the late 19th century focused on mammary gland development in human embryos [2–9], comparative anatomists interested in ontogeny and phylogeny also examined monotremes and marsupials [10–14], reviewed in [15]. The rabbit embryo was a useful model as well [2, 16–21], as rabbits have the practical advantages of greater availability than human specimens; of ovulating upon mating which allows precise timing of progress of the pregnancy; of producing several embryos of the desired stage in one pregnancy; and of a relatively short gestation period of 30 days. Furthermore, rabbits resemble humans in the continued mammary gland development in male embryos, while in male mouse embryos, mammary glands are destroyed by testosterone signaling [22–24]; as well as in the formation of several primary milk canals per gland, as opposed to one in mouse, as described below.

In the second half of the 20th century, the focus within mammary gland research shifted towards postnatal development, lactation and breast cancer. Only a few groups continued to study embryonic morphogenesis of mammary glands. But the field regained greater interest in the past two decades, after the advent of genetic engineering techniques for mammals, most widely applied in mice. Since then, a considerable number of genes have been identified as regulators of select aspects of embryonic mammary gland development in mice, as reviewed throughout this issue [25–31]. As a result, more insight has been gained in mammary morphogenesis

itself, and in its parallels with breast cancer [32, 33]. This review will focus on morphogenesis of embryonic mammary glands, hereafter referred to as mammary rudiments (MRs), in mouse and rabbit embryos. It includes cellular processes and tissue interactions involved in morphogenesis, and serves as a basis for the detailed description of the role of several key molecules and genetic pathways in separate reviews elsewhere in this issue [25–31]. Due to space constraints, we refer to previous related reviews for in-depth description of morphogenetic events [22, 34–37] and here present generalities besides a focus on new insights of the last decade.

Ontogeny of Mammary Glands

Mammary glands are believed to have formed relatively late in evolution, and to derive from skin glands that produced nutritious secretions which replenished evaporating nutritious liquids from the parchment-shelled eggs of synapsids [38–41]. The contiguity of mature mammary glands with the skin led to the contention that the glands have a surface ectodermal origin. This contention was supported by findings in embryos of monotremes, marsupials and eutherians, in which the early rudimentary mammary gland existed as a local thickening within the surface ectoderm or as an epithelial bud within the maturing epidermis, without clear boundary between mammary rudiment and ectoderm or epidermis [15]. Indeed, culture of heterotypic tissue-recombinations of mouse embryonic flank mesenchyme with surface ectoderm of a rat embryo gave rise to branched epithelial structures consisting solely of rat cells, indicating the pure ectodermal origin of the glandular epithelium [42].

A Quick Glance at, and Nomenclature of, the Stages of Embryonic Mammary Gland Morphogenesis

While the number of mammary glands varies widely among the various mammalian species [43], the glands are most commonly present in left-right symmetrically located pairs. Their location is ventral, usually at the thorax (e.g. in primates) or inguen (e.g. in ungulates) when the number of glands is low, but ranges from the axilla to the genital tubercle when higher numbers of mammary glands are present, e.g. in pigs.

An imaginary, fluent, slightly curved line can be drawn through the positions of the mammary glands on one side of the adult body, and is referred to as the mammary line (ML). Mammary lines come in pairs, with one line present on each ventro-lateral boundary of the body. In some species, such as the rabbit, MLs become anatomically visible as elevated ridges in the surface ectoderm (i.e. the precursor tissue for the epithelial component of the skin), one on each flank of a 13 day old (E13) embryo [19]. Four or five mammary glands develop at distinct and precise locations from and within each ridge (Fig. 1). It was therefore long believed that the ML exists prior to ontogenesis of the mammary gland. However, recent investigations of the formation of the MLs and five pairs of mammary glands in mouse embryos [44] and overlooked data from rabbit embryos [18] suggest otherwise. While this controversy will be discussed in greater detail at the end of this review, the current section will briefly focus on the nomenclature of the sequential stages of embryonic mammary morphogenesis as described for mouse [22] and rabbit [45].

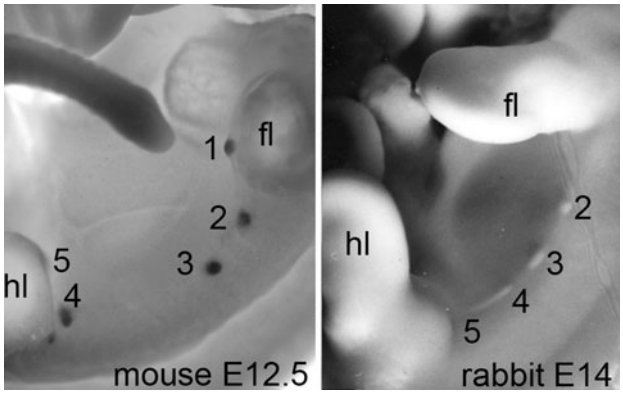

Fig. 1 Position of the mammary rudiments in mouse and rabbit embryos. Lateral view of flanks of a mouse (*left*) and rabbit (*right*) embryo. MRs are visualized by hybridization to *Wnt10b* (mouse, C57Bl/6) or elevation as observed in scanning electron microscopy (rabbit), and numbered in anterior-to-posterior sequence. The MR1 in rabbit is hidden behind the forelimb. Abbreviations: fl: forelimb; hl: hindlimb (forelimb removed from the mouse embryo)

The nomenclature used to describe the stages of embryonic mammary gland development has been inconsistent and difficult for the non-specialist to comprehend. We would therefore like to suggest the implementation of a standardized terminology for subsequent scientific literature, with mouse studies particularly in mind. The mouse embryonic mammary glands are usually numbered as pairs MR1 through MR5 in rostro-caudal (or antero-posterior) order (Fig. 1), although there are arguments in favor of their individual numbering from 1 to 10 [46]. In reference to the incompletely developed embryonic mammary gland at no particular embryonic age or developmental stage, thus encompassing all embryonic/fetal developmental stages, the words mammary ***primordium (primordia)***, mammary ***anlage(n)*** or mammary ***rudiment(s)*** (MRs) can be used. In the existing literature, these words are generally used in reference to the epithelial compartment of the embryonic mammary gland, because MRs initially consist of an epithelial component only. However, it is imperative to bear in mind that the dermal mesenchyme directly adjacent to the mammary

epithelium, as well as the subdermal mesenchyme, both undergo important changes as development proceeds, which are required for morphogenesis of the epithelium through continuous reciprocal epithelial-mesenchymal interactions, as discussed in several papers in this issue [25–28, 30, 47, 48]. These mesenchymal tissues are thus essential components of the mammary gland as an organ and remain so during postnatal life. They should therefore be included when one refers to a mammary primordium, anlage or rudiment. There is an additional stage-specific nomenclature, derived from the shape of the epithelial compartment of the embryonic mammary organ.

The first evidence of formation of the mammary primordia proper along the ML is the emergence of elliptical pseudostratified multilayered structures, called ***placodes***, within the otherwise single-layered basal layer of the ectoderm. The placodes become visible in rabbits after E13, and in mice, usually between E11 and E12. The precise embryonic age at which the placodes appear differs per strain, and furthermore, the pairs develop asynchronously (as discussed below). Therefore, the ages indicated in Fig. 2 are to be taken as approximations. Consequently, when reporting phenotypes based on histology, it is often relevant to perform complete serial sectioning and to refer to observed versus expected morphogenetic stages of mammary gland development in addition to the embryonic age. It is furthermore important to indicate which MRs are affected and shown, given the differences in genetic and morphogenetic programs for each MR, as discussed below and in [46].

As shown in Fig. 2, the ***placodes*** transform via a hemispherical ***hillock*** (mouse E12.5, rabbit E14) into a spherical structure, referred to as a ***bud*** (mouse E13, rabbit E16). By then, several layers of dermal mesenchyme adjacent to the bud have condensed and aligned from a random into a concentric orientation, as morphological evidence that these cells have differentiated into the primary mammary mesenchyme (MM). At around that same time, the *surface ectoderm* of the flank has generated a suprabasal layer on top of the basal layer indicating the maturation towards *epidermis*. Some buds will then sink

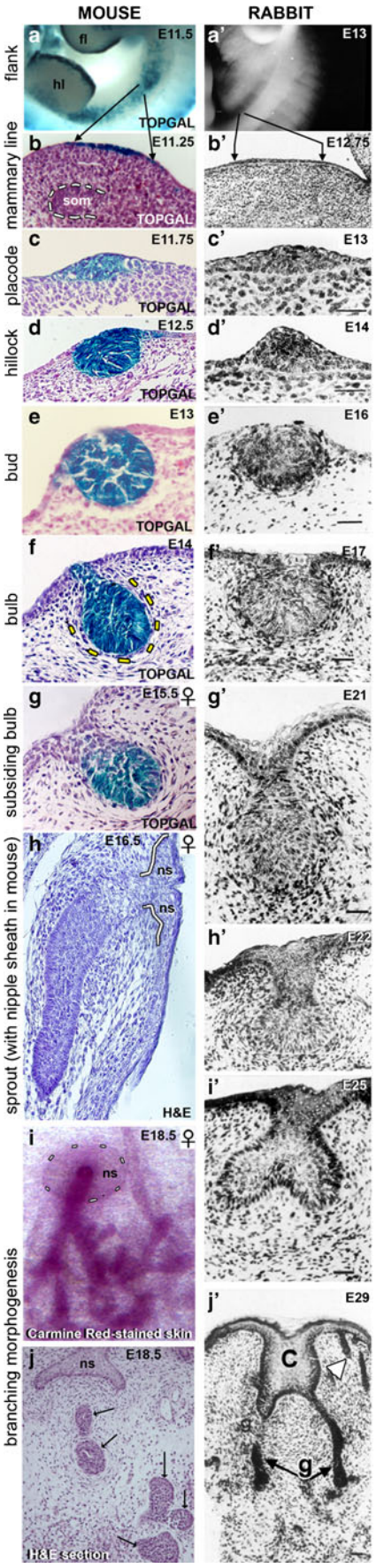

Fig. 2 Histological analysis of subsequent morphogenetic stages of mammary rudiments in mouse and rabbit embryos. Mammary nomenclature and embryonic (E) stages are indicated. **a** Flank of a mouse embryo subject to whole-mount staining for TOPGAL-F ([49], hereafter named TOPGAL), shows the mammary line in an advanced stage as a blue band between fore- and hindlimb; **a'**: Scanning electron micrograph of a rabbit embryo flank, showing the mammary ridge as an elevation between fore- and hind-limb. *Arrows* from **a** and **a'** to **b** and **b'** are indicating the width of the mammary streak/line, visible as blue (TOPGAL^{+ve}) cells in the mouse embryo prior to E11.5, and as enlarged cells in the E12.75 rabbit embryo prior to elevation as a ridge. In B-G, ME of MR3 is visualized by *blue staining* for TOPGAL in mouse embryos. *Yellow dashed line* outlines MM in F. *White lines* outline nipple sheath in H. *Arrows* in J point to galactophore ducts, and *white arrowhead* to a hair follicle. **b'**–**j'**: H&E staining of sections through rabbit MRs. Abbreviations: c: cistern; g: galactophore duct; H&E: Hematoxylin/Eosin staining; ns: nipple sheath

deeper into the dermal mesenchyme and become constricted in the neck region where they connect to the epidermis. Based on the resemblance to a light bulb, this morphogenetic stage is often referred to as ***bulb*** stage, typically observed in MR3 of mouse embryos at around E13.5, rabbit at around E17. In male mouse embryos, the bud then undergoes testosterone-mediated destruction [23–25, 50]. This phenomenon is typical for mice and rats [37, 51]. In most mammals, mammary development continues in males, but does not progress beyond producing a rudimentary ductal structure, due to the absence of female steroid hormones. In female mouse embryos, the mammary primordium enters ***sprout*** stage at around E15.5, when the distal end of the bulb elongates into the deeper lying mesenchyme, i.e. the secondary mammary mesenchyme, also referred to as the fat pad precursor (FPP) tissue. Some primordia transit from bud stage to sprout stage without an intermediate bulb stage. Subsequent branching morphogenesis of the sprout produces a small ***glandular tree*** by E18.5, i.e. one or several days before birth depending on strain. In rabbit, the spherical part of the bulb enlarges and elongates between E17 and E23 and also enters a deeper zone of mesenchyme that contains adipose cells. At E26, the bud starts to lobulate and bi- or trifurcate. Each of these secondary buds gives rise to a primary milk canal which undergoes branching morphogenesis [45].

In both mouse and rabbit embryos, hair follicle formation starts in the epidermis well after the onset of mammary gland formation. No hair follicles form in the immediate vicinity of the MR, due to inhibitory signals of the mammary mesenchyme [52, 53] that are also required for the formation of nipple tissue at around E16.5 in mouse embryos [54, 55].

Asynchrony and Inter-Independence

The mammary primordia in mouse embryos appear asynchronously in the course of a half to full day. Initially, the order of their formation was believed to be first MR3, then MR4, soon followed by MR1 and MR5 which emerge simultaneously, and finally MR2, as determined by assessments by scanning electron microscopy of mouse embryos between E11 and E12.5, which visualizes the slight elevation of the placodes above the surrounding surface ectoderm [56]. This order was confirmed by identification of the placodes by whole-mount in situ hybridization with *Wnt10b*, which marks formation of the mammary line and placodes [44]. However, use of other molecular markers suggests a slightly different temporal order of mammary primordia formation [57], and unpublished histological analysis of C57Bl/6 mice in the Howard-lab and Veltmaat-lab indicates that the MR1 may sometimes emerge at around the same time as or slightly after MR3, but prior to MR4. Although the observed order may depend on the method of analysis or vary per strain, there is agreement that MR3 emerges first and MR2 last. This indicates that the mammary rudiments develop neither in anterior-posterior nor in dorsal-ventral order, unlike other repetitive structures such as somites, teeth and feathers. In rabbit, the temporal sequence in which the MRs appear has not been studied at the same level of detail, although later stages of development are believed to occur in anterior-posterior order. However, it must be noted that this is true for the MRs that are visible on the flank, of which the anterior-most MR is similar in position to the mouse MR3. Notably, the rabbit also has a pectoral/axillary MR whose timing of formation and developmental progress has not been well-reported, but may fall in between that of those on the flank, similar to MR1 in the mouse embryo.

In mouse, the MR pairs develop at dissimilar speeds such that MR2 catches up with MR3 by E13.5 [58]. The three thoracic MRs sprout by E16.5, followed by the two inguinal pairs at E17 [37] and MR2 is more extensively branched than MR3 by E18.5 [22]. One can speculate about the factors that bring about these changes, such as e.g. differences in proximity to the thoracic artery as a source of growth factors, but the strict adherence to the temporal program of development of MRs when cultured ex vivo, argues against an influence of hormonal and other systemic factors [59], and in favor of MR-intrinsic properties controlling the speed of development. These differences indicate that the various pairs of mammary glands are not identical copies of one another, and may use different mechanisms for their individual development and growth [22]. This idea is well supported by studies of wild type and mutant mice in which one or several MR pairs are not induced or sustained, in non-linear combinations [22, 33, 46, 60]. These studies indicate that each pair of MRs develops independently of the other pairs, and that the individual pairs differ in their molecular requirements for induction and maintenance.

Tissue Interactions and Cellular Mechanisms of Embryonic Mammary Gland Development

Mammary gland development relies on continuous reciprocal epithelial-mesenchymal interactions [35, 61]. The known molecular mechanisms that mediate such interactions or otherwise contribute to generating a mammary cell fate (be it epithelial or stromal) are described in detail in separate reviews of this special issue. Here, we will restrict the focus to changing cell behaviors that contribute to mammary morphogenesis.

Growth

Prior to the formal evidence for the ectodermal origin of mammary epithelium (ME) [42], tissue recombination studies in rabbit and mouse had revealed that the capacity to form ME was not intrinsic to the surface ectoderm, but induced by the dermal mesenchyme [21, 59]. The nature of the inducing molecules from the mesenchyme were

unknown, but in 1973, Propper proposed epidermal growth factor (EGF) as a candidate [18], and interestingly, three decades later the EGF family member Neuregulin3 was found in the dermal mesenchyme underlying MR3 in mouse embryos, where it likely plays an inductive role [27, 62].

Several studies focused on behavioral differences between surface ectodermal and mammary epithelial cells to uncover cellular mechanisms of mammary line and placode formation. Balinsky compared the fraction of cells in mitosis between pooled ME from E11 to E14-stage mouse embryos or equivalent developmental stages in rabbit, and pooled ectoderm/epidermis of those stages. He concluded that MRs do not grow by enhanced cell proliferation, and suggested that ectodermal cells migrate centripetally towards the growing placodes [17, 20]. In a later study, cell behavior was assessed by means of ^{3}H-TdR-incorporation in DNA at E13. At this bud stage, the ME shows very little to no incorporation of label, thus no proliferative activity, greatly in contrast to the epidermis. Chasing at 24 h post-pulse showed the presence of ^{3}H-TdR^{+ve} (thus epidermally-derived) cells in the neck of the ME, which transformed the MR from bud to bulb during that day [35]. This indicates that ME grows by accretion of epidermal cells.

These latter results were also interpreted as a 24-h proliferative quiescence within the ME between E13 and E14, a notion which has been propagated in subsequent literature. However, given the rapid morphogenetic changes that occur in those first days of mammary development, and the emerging unique identities of each of the five pairs of mammary glands in mouse, cell behavior was analyzed per MR pair and at each day separately from E11.25 to E13.5 by pulse-chase analysis of BrdU-incorporation in a recent study [58]. With small differences between the MRs, overall the ML and MRs have very low to no proliferative activity between E11.25 and E13.5. Thus, the proliferative arrest between E13 and E14 is not temporary. Instead, cell proliferation is almost absent in ME from E11.25 onwards and does not contribute to growth until at least E14.

Between E11.25 and E12.5, ectodermal influx is the major determinant of MR growth. Between E12.5 and E13.5, cuboidal to columnar hypertrophic transformation of the basal cells of the ME becomes an additional important contributor to MR growth [58], followed by epidermal influx to contribute to formation of the neck between E13 and E14 [35]. Given that the entire ME is TOPGAL-positive at that time (Fig. 2f), while there is no TOPGAL- or *Wnt10b*-positive mammary line visible anymore after E12.0 (Fig. 4), these influxed cells seem to engage in Wnt-signaling only upon arrival in the MR.

DNA replication resumes at E14.5 within the ME, but it takes until 16 for the sprout to grow out [35]. Balinsky analyzed E16 to E19 stages by comparing the mitotic index of the proximal (i.e. connected with the epidermis) versus distal (i.e. residing in the fat pad precursor) ends of pooled MRs, and found a significantly higher proliferative activity in the distal part of these MRs, consistent with their sprouting/branching activity [20]. Given the differential growth among MRs, it would be of interest to determine proliferative activity for each of the MRs individually for these stages as well.

Morphogenesis

In the rabbit embryo, the ML becomes an elevated ridge in the ectodermal landscape of the flank at E12 upon fixation, or in a fresh embryo at E13. As the mammary placodes transform into hillocks, the ridge continues to exist for at least half a day, until around E13.5-E14 it subsides in between MRs and leaves the bud-shaped MRs behind as initial escarpments. These buds transform into bulbs and subside by E15.5, such that they are no longer externally visible [34] (Fig. 2). In mouse, similar events of elevation and subsidence occur, with the difference that the ML is not obviously raised as a ridge, but the MRs are elevated above the surface ectoderm at hillock stage and bud stage until about E13.5 [56], but subside before E14.5 when they transform into bulbs. What regulates this elevation and subsidence, as well as the shape changes of the growing MRs, is not known.

Meanwhile, at E12.5-E13, a few layers of dermal mesenchyme directly adjacent to the ME condense and line up in rather concentric rings around the mammary bud, to form the primary mammary mesenchyme (MM). Epithelial-mesenchymal interactions mediated by Wnt signaling seem required for this initial condensation in male and female mouse embryos [63]. Further male-specific condensation of the MM depends on peptides produced by the ME, that elicit signaling and androgen receptor (AR) expression in the MM. Activation of AR by testosterone in males leads to constriction of the MM as well as cell death within the MM and ME. This reaction is especially strong at the proximal area of the MR, where the MM is broader, and leads to disconnection of the distal part of the ME from the skin epithelium [50, 64]. The distal part may survive and grow without outlet to the skin.

In females, the next morphogenetic event is neck formation in the ME. According to the ^{3}H-TdR tracing experiments in mouse, mentioned above, the neck seems to consist of newly accreted epidermal cells [35]. This seems to be supported by the absence of TOPGAL in both epidermis and neck of the ME at E15.5, in contrast to TOPGAL expression in the bud-region of the bulb (Fig. 2g). Despite the absence of TOPGAL in these cells, Wnt signaling is required for MR growth, neck formation and eventually maintenance of the MR [30, 63]. We speculate the absence of TOPGAL expression in the neck may be a first indication that these cells are prospective nipple sheath instead of mammary cells, and become the pale cells in the nipple sheath area (proximal end of the sprout) at E16.5 (Fig. 2h). Similarly, the cells in the neck of rabbit MRs

keratinize at the same time as epidermal cells, which most likely attests to their epidermal origin [45].

In the mouse, sprouting occurs via upregulation of proliferative activity [20, 35] and depends on interactions with the MM that are themselves initiated by peptides from the ME that activate receptors in the MM [25]. The tip of the bud breaks through the basal lamina, that is well defined around the ME at bud stage, but becomes less distinct at around E16, allowing the sprout to enter the underlying fat pad precursor (FPP) [65]. At around the same time, the MM also signals to the overlying epidermis, which triggers nipple (sheath) formation [54, 55]. Note that in the rabbit, the nipple does not form until after birth [18].

In E26 rabbit embryos, the mammary bud starts to lobulate and bi- or trifurcate. Each of these secondary buds gives rise to a primary milk canal which undergoes branching morphogenesis [45]. This phenomenon resembles morphogenesis of the human breast and contrasts with the sprout in mouse giving rise to a single primary milk canal which undergoes branching morphogenesis. Branching morphogenesis of the mammary sprout in the mouse embryo was proposed to start by bifurcation, as inferred from the presence of a shallow cleft at the distal end of the bud during its elongation into a sprout at E15.5 while the first branches are not visible until E16.5-E17 [65]. However, dissected epithelia of E17.5 mouse MRs (Fig. 3), suggest the first branch can form by side branching, as inferred from the small bud on the sprout of MR1. Although the equal length of some branches of the more advanced MRs2-5 suggest these branches may have arisen via bifurcation, side branches can be seen budding off from existing branches in MR2, MR4 and MR5. Thus, the earliest branching events are probably a mix of bifurcation and side branching. The basal lamina around the ME becomes less distinct at E16, and remains less distinct at the tips of the branches at E17 and E18 [65, 66], as if to allow invasion of the new branches into the FPP. While branching morphogenesis requires budding and elongation of new branches, and several molecules in the FPP have been identified to induce the first branching events in the MR in mouse [28, 29, 56], much is still to be learned about the details of mammary branching morphogenesis. It is not stereotypical, in contrast to branching of organs such as lung and kidney. Thus, the interplay between inductive and inhibitory factors for budding and branching may be more complex than in those other organs.

Both in mouse and rabbit embryos, lumen formation in the mammary gland starts prior to birth [45, 65]. As soon as branching morphogenesis starts, intercellular spaces begin to develop by cell death within the new branches. While the nipple sheath is forming, small intercellular spaces appear in the neck of the MR, indicative for lumen formation at this proximal end of the sprout as well. The lumen then connects to the funnel-shaped indentation [65] that is visible in the epidermis at the sites where the mammary sprouts reside as early as E15.5 in mouse embryos [58], prior to nipple sheath formation [54]. Before birth at E19-E20 depending on strain, the discontinuous lumina have fused to form one continuous ductal system [65].

Acquisition of and Commitment to a Mammary Cell Fate

The ectodermal potential to acquire a mammary epithelial identity is normally initiated at the time of mammary line and placode formation between E10.5 and E11.75, but placode formation can occur later as evidenced by the appearance of MR3 at E12.5 instead of E11.25 in *Pax3*$^{ILZ/ILZ}$ (null) mutants [44, 67]. MRs can even be induced in tissue-recombinants of E13 rat dorsal epidermis with E13 mouse mesenchyme [42]. Note that at these embryonic stages, the epidermis has not yet stratified or formed hair follicles, and it would be of interest to know whether after these events, the

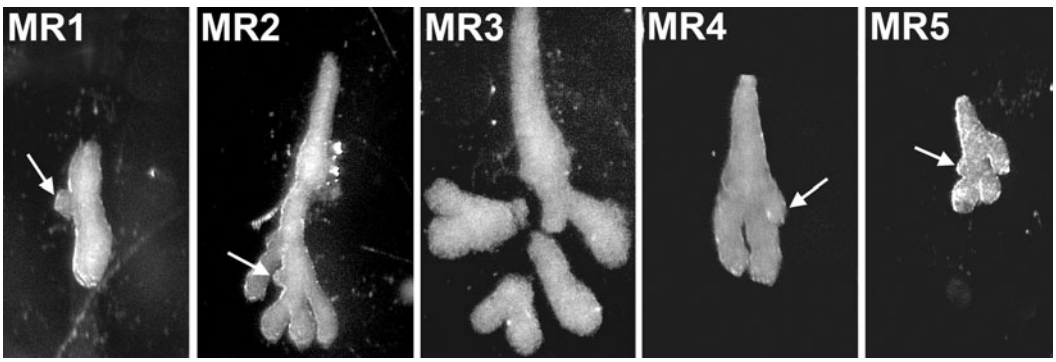

Fig. 3 Branching morphogenesis of murine mammary epithelia at E17.5. Epithelia of mammary rudiments (MR) 1 through 5 were isolated via trypsin/pancreatin enzymatic tissue-separation and presented at same magnifications. Note the differences in size and branching morphogenesis. *Arrows point* to side branches budding off from existing branches, suggesting side branching is an early contributor to mammary tree formation

epidermis can still be induced to form mammary epithelium. Whether all ectodermal cells along the ML maintain their acquired mammary potential is arguable, because seemingly excess cells at the terminal part of the mammary ridge in rabbit embryos undergo necrosis [18]. In mouse embryos, *Wnt10b* expression disappears along the mammary line while the MRs grow [44], and it remains to be determined whether that is due to *Wnt10b*$^{+ve}$ cells being integrated in the growing MRs, dying, or reverting to an epidermal fate (Fig. 4). In MRs proper, a mammary fate seems to be established almost immediately, because E12.5 and E13.5 MRs, when isolated and transplanted into cleared mammary fat pads of 3-week old female mice, can grow out to resemble a normal mammary gland [68, 69], while embryonic lung, pancreatic or salivary epithelia cannot [69]. Recently, insights have been gained in the molecular signals that may mediate such fate determination [70].

Commitment to this fate is certainly established prior to E16.5, as evidenced by milk protein production in recombinants of E16.5 mammary epithelium with salivary mesenchyme, grafted under the kidney capsule of female mice treated with pregnancy hormones [71]. Notably, E15.5 seems to be crucial stage for maintenance of a mammary epithelial fate, as in the absence of *Lef1* or *Msx1/2*, MRs regress or revert to an epidermal fate at this timepoint [63, 72, 73]. It is of interest that this happens just before keratinization of the epidermis at E16.5, perhaps suggesting that cells that are by then not fully committed to a mammary fate respond to epidermis-inducing signals. Moreover, this is also the stage at which the regenerative potential of ME dissociated into single cells and grafted into cleared fat pads, increases drastically, from almost absent at E15.5, to well measurable at E16.5, and increasing further at E18.5 [68, 74].

Time to Revise Some Long-Standing Concepts?

In the Rabbit Embryo, Mammogenesis Starts Prior to the Emergence of a Mammary Ridge

As mentioned previously, mammary placodes emerge along a mammary line on each side of the body. In some mammalian species, notably the rabbit, this line is anatomically visible as an elevated ridge at E13.5. This ridge undergoes fragmentation in the anterio-posterior direction. The resulting fragments subside but leave behind elevated streak-like segments that eventually form into round buds [34, 35]. As no elevated ridge was observed in mouse embryos, the formation of a mammary line in mouse embryos was considered controversial [75–77] and the onset of mammary gland formation was consequently believed to be essentially different between mouse and rabbit. Nonetheless, a streak of expression of *Wnt10b* (encoding a secreted factor) on the mouse embryonic flank was assumed to mark a mammary line [78]. In-depth analysis showed that *Wnt10b* co-localizes with areas in the surface ectoderm where cuboidal cells enlarge to become columnar and a suprabasal cell layer arises, including at the sites where the five pairs of mammary rudiments will form [44, 67]. It now becomes of interest that along the line of the prospective elevated ridge in E12.75 rabbit embryos, cell enlargement and multilayering are also observed (Fig. 2b') [18], similar to what occurs in mouse embryos [44, 67]. Furthermore, at the time when the mammary line in rabbit embryos is visible as an elevated ridge, the mammary rudiments have already acquired a bud shape, indicating that the mammary ridge represents not the initial, but an advanced stage of mammogenesis. In mouse embryos, the mammary rudiments do become slightly elevated between E11.5 and E12.5 [56], in that sense resembling residues of a ridge. Together, these data suggest that the onset of mammogenesis in the rabbit and mouse resemble each other more closely than assumed so far.

The Mammary Line, a not so "Commonplace" for Mammary Gland Development

Despite a call already in 1976 for more nuanced thinking about the mammary line and its relationship to the mammary rudiments [34], it is generally thought that the mammary line is a structure that extends from axilla to inguen, existing prior to MR formation, and from and within which all MRs develop. However, the two inguinal pairs of MRs in rat form without connection to the mammary line, and similarly, the pectoral MR in rabbit forms from a crest that is never attached to the mammary ridge [17, 18, 34]. The identification of *Wnt10b* as a molecular marker for the murine mammary line has revealed that in mouse embryos, the mammary line does not pre-exist as a continuum prior to MR formation [44]: At mouse E10.5, expression of this marker descends as a thin line from dorsal to the forelimbs toward the abdomen, and reaches the level of the diaphragm or approximately the 16th somite by E11.0. Just anterior to that level, the line of *Wnt10b* expression becomes fragmented [44] (Fig. 4a). While *Wnt10b* expression levels increase at that level as an indication that a mammary rudiment (MR3) will form at that position, fragments with a lower expression level appear progressively posterior to that level, down to the inguen. In the last supra-inguinal fragment, *Wnt10b* expression increases to indicate the onset of formation of another mammary gland (MR4). Meanwhile, the other fragments between them appear to fuse to become a continuous line [44] (Fig. 4). Unpublished data have revealed similar expression kinetics for the transgenic reporters TOPGAL-F [79] (Veltmaat; Kogata and Howard) and sSHIP-GFP [80] (Kogata and Howard). Notably, expression domains of any of these markers fuse into one continuous line only ***after*** the formation of morphologically distinct MR3 and MR4.

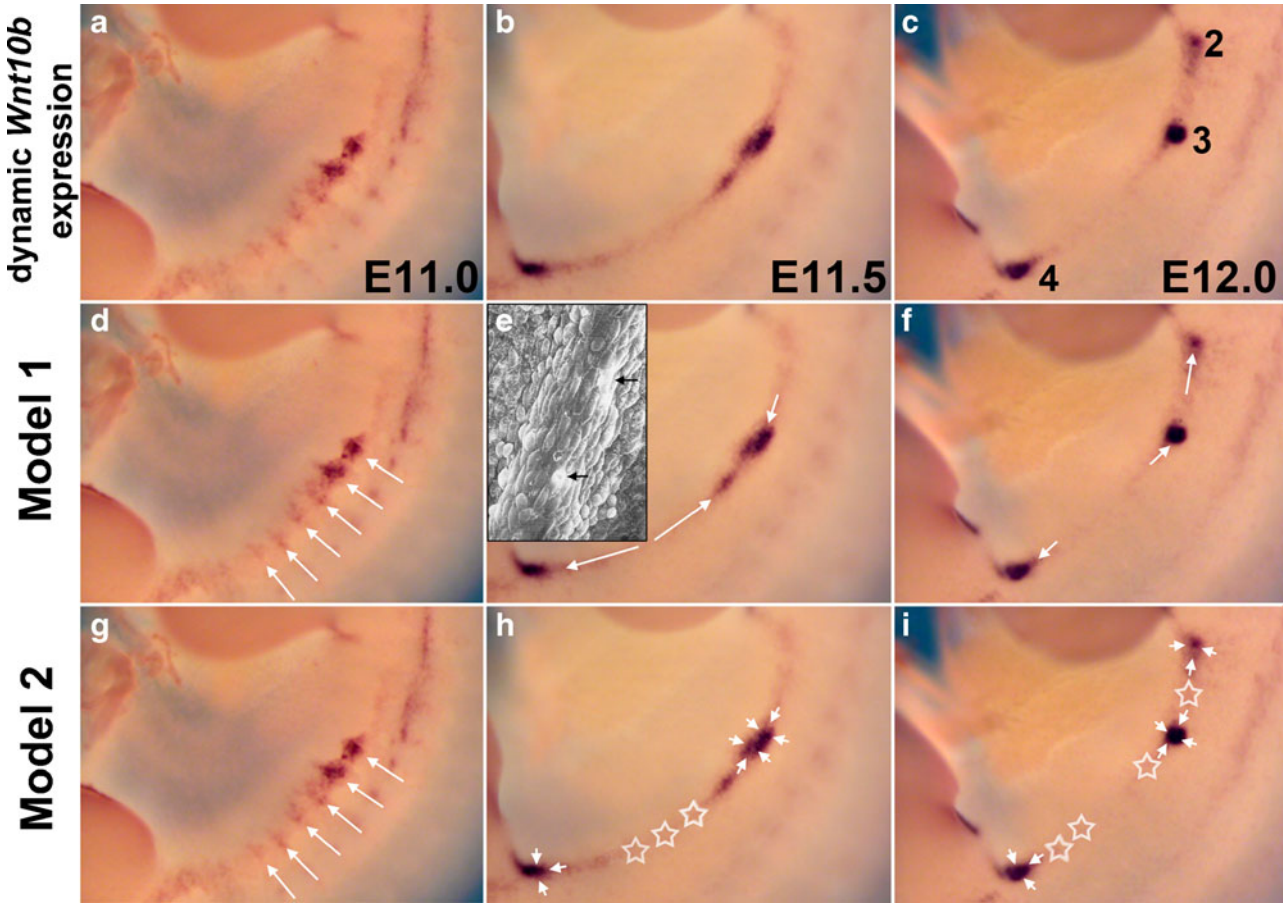

Fig. 4 Models for cell migration during ML and MR formation. *Top row* shows mammary streak formation on the mouse embryonic flank, as visualized by hybridization with *Wnt10b* probe (with permission modified from [44]). MRs are indicated by number in panel **c**. Panels **d** and **g** depict the assumed dorso-ventral migration leading to mammary streak formation on the flank, based on involvement of somitic signals [67]. In current thinking (Model 1, *middle row*) this is followed by cell migration along the mammary streak/line, contributing to formation of MR2, MR3, and MR4. This model is based on loose interpretation and extrapolation of observed spindle-like cells on the edge of the mammary ridge in rabbit (*arrows* in inset in panel E, reproduced with permission from [19]). Model 2 (*bottom row*) presents an alternative model featuring regions of centripetal migration contributing to MR formation, and regions where induced mammary potential is lost along the ML. *Arrows* indicate migration, stars indicate loss of mammary potential in cells. See main text for detailed explanation

The line then extends upward towards the axilla, where formation of another mammary rudiment (MR2) becomes apparent in the sub-axillary position. During these events on the flank between fore- and hindlimb, two separate streaks of *Wnt10b* expression form in the axilla and inguen [44], as also noted for TOPGAL-F and sSHIP-GFP expression. These streaks are initially unconnected to the line on the flank, yet each give rise to a mammary gland; MR1 in the axilla, MR5 in the inguen. The formation of these latter MRs at positions that are initially not visibly connected to the mammary line on the flank, resembles the formation of the pectoral (axillary) mammary gland in rabbit or inguinal glands in rat, whose positions are unconnected to the ridge on the flank [34]. These observations were often overlooked in subsequent literature, perhaps because it contradicted the belief that the mammary line or ridge is a continuous structure on and from which all mammary glands form.

Thus, the mammary line in mouse is initially evident (as assessed by gene expression pattern(s) in whole embryos), not as a continuum, but instead consists of three distinct and independent streaks of expression of endogenous *Wnt10b* mRNA or transgenic s-SHIP-GFP or TOPGAL-F, on which MRs appear prior to fusion of the three streaks into one continuum. One streak is present in the axilla, one on the flank, and one in the inguen. Perhaps the streak on the flank has to be considered as two streaks [46]. It is notable that the 'junctions' between the streak(s) on the flank with the axillary respectively inguinal streak are angular [44]; in other words, once the streaks have fused to a continuous mammary line, this line is not as smooth and slightly curvilinear as the imaginary mammary line that one can draw through all positions where mammary glands may form within a species. We can conclude that, contrary to the commonly-held notion, the mammary line is not a pre-

existing common site of presumptive mammary rudiment formation, but in the mouse forms from (at least) three independent streaks, and concomitant with mammary placode formation. These separate streaks for MR induction may lie at the basis of an explanation for the individual identity of each of the MR pairs [46].

Directionality of Cell Movements During Mammary Line and Rudiment Formation

As mentioned previously, Balinsky suggested that in mouse embryos, MRs form by centripetal aggregation [20]. In rabbit embryos, the involvement of cell migration towards the placode position was inferred from the observation of spindle-like cells on the top edge of the mammary ridge, many of which are polarized along the length of the ridge (insert Fig. 4e) [19]. Moreover, experiments in which charcoal was deposited on the mammary ridge or outside, and traced after 24–48 h, indicated that only cells from the mammary ridge but not from the adjacent epidermis, are contributing to MR growth [18]. In subsequent literature, these data seem to be loosely interpreted and extrapolated to mouse embryos, as if MRs are formed and grow by accretion of cells that migrate along the length of the mammary line toward MR positions (Fig. 4e–f, Model 1). However, it is notable that in rabbit, mammary buds were proposed to individualize from the mammary ridge by local contractions of the ridge that are predetermined in the mesenchyme, at positions where epidermal cells seem to migrate centripetally and assemble into spherical buds [19], much in accordance with Balinsky's model [17, 20]. In rabbit, the mammary ridge itself is formed prior to morphogenesis of the individual MRs, and with exception of the pectoral MR, the ME accretes cells from the ridge proper, but not from the adjacent epidermis [18]. By contrast, recent data suggest that in mouse embryos, ectodermal cells from outside the mammary line are accreted into the MRs [58]. Thus, for the formation of the mammary streak on the flank, and MR2, MR3 and MR4 on that streak, we propose Model 2 of alternating regions of centripetal migration contributing to MR formation, and regions where induced mammary potential is lost (Fig. 4g–i), as follows:

1) At E11.0, the mammary streak on the flank presents as a fragmented line of intense *Wnt10b* expression overlying the hypaxial (ventral) tips of the somites; the fragments having dorsal extensions with lower *Wnt10b* expression levels, overlying the length of the somites [44] (Fig. 4a). As such, this fragmented pattern mirrors the segmentation pattern of the underlying somites, suggesting the somites may play an inductive role in formation of these fragments comprising the streak, and thus the mammary glands rudiments that arise on/from it. As the somites give rise to the dermal mesenchyme, this suggestion would be in agreement with the notion that a mesenchymal factor or factors induce mammary gland formation [21, 59].
2) *Fgf10* expression in the somites, and particularly its high expression level in the hypaxial dermomyotome of the somites between E10.5 and E11.5, is required for induction of the mammary streak on the flank, with exception of the small posterior end of the streak where MR4 will form [67]. The gradients of *Fgf10* expression within and among somites are mirrored in the gradients of initial *Wnt10b* expression among the fragments along the anterior-posterior axis of the flank as well as the dorsal extensions of these fragments. FGF10 (indirectly) induces ectodermal *Wnt10b* expression via activation of its own main receptor, FGFR2-IIIb which is expressed in the ectoderm [67].
3) The somites are located adjacent to the neural tube at E10, at which time they do not yet express *Fgf10*. As the somites start to elongate at around E10.5, they start to express *Fgf10; Fgf10* expression increases while ventral elongation proceeds. The kinetics of *Wnt10b* expression in the ectoderm suggest that *Wnt10b*$^{+ve}$ cells are dragged along with the elongating somites towards the position of the prospective ML (Fig. 4d, g). ML position is determined by the end of hypaxial elongation of the somites [67]. This model would be consistent with chemotactic properties of *Fgf10* found during formation of other organs, e.g. lung [81].
4) From the moment the mammary streak is histologically visible as a band of enlarged cells in the surface ectoderm, its cells have little to no proliferative activity, and thus none or few of them incorporate BrdU, in contrast to the high levels of BrdU-incorporation in the adjacent ectoderm [58]. Yet when BrdU^{+ve} cells are chased at 24 h after the pulse, a significant proportion of them have been recruited into the ME in these 24 h [58]. As the mammary streak contained few to no BrdU^{+ve} cells at the time of labeling, the BrdU^{+ve} cells that ended up in the MRs must have resided outside the mammary streak at the time of labeling. During the next 2–3 days, ectodermal cells are still recruited into the MRs [58] even if the mammary streak/ML is not evident anymore (Fig. 3c). While this may seem to contrast with the phenomenon observed in rabbit embryos, in which MRs only recruit cells from the mammary ridge and not from adjacent epidermis, note that the rabbit's mammary ridge contains more cells than the murine mammary line, and moreover becomes elevated at a time when MRs are already at hillock/bud stage.
5) Instead of migrating along the ML toward the MRs (Model 1), it is more likely that cells migrate in a centripetal manner towards the positions of the MRs, as proposed previously [19, 20] (Model 2). The

gradients of *Fgf10* within and among the somites #12-#18 may mediate centripetal cell migration at the level of MR2 (above somite #12) and MR3 (above somite #15-#16) [67], and another somitic signal may be involved at the level of MR4.

6) Taken furthermore into account that the ML is ***not*** a pre-existing structure, one could consider the individual *Wnt10b* fragments along this part of the ML in E11.0 embryos (Fig. 4a) as individual sites where MRs can arise (and do for example in pigs). Along this line of thinking, the mammary fate is not maintained at sites where appropriate signals are not sufficiently present, while this fate is sustained in the presence of sufficient appropriate signals, which can e.g. be mimicked by elevated ectodermal Wnt or NFκB signaling [82–84].
7) In the areas between the MRs, *Wnt10b* expression may be lost due to cells losing their acquired mammary potential (stars in Fig. 4h, i), either by reversion to an ectodermal/epidermal fate, or by necrosis. In support of the latter proposal, necrosis is observed in seemingly excess cells at the terminal part of the mammary ridge in rabbit embryos [18].

Concluding Remarks and Outstanding Issues in the Field

In this review, we have presented the morphogenetic phases of embryonic mammary development in the mouse and rabbit. We propose standardization of nomenclature, to facilitate better comparison of published phenotypes. Preferably, the presentation of mammary phenotypes should combine mammary morphogenetic stage with embryonic age and include the position (by number) of the MR, because of the developmental asynchrony among mammary rudiments within one embryo as well as across strains.

While mammary morphogenesis relies on continuous reciprocal epithelial-mesenchymal interactions, the molecules in these interactions are not reviewed here, as they will be discussed in detail in several other articles in this issue. Several genetically modified mice show phenotypes in non-overlapping subsets of MRs, indicating that each MR has its own sensitivity to loss of certain genes. Preliminary expression profiling data support that this may relate to unique differential expression (in the order of tens of genes) for each MR pair [Sun and Veltmaat, http://www.veltmaatlab.net/research.html#sunli]. This distinct identity for each MR will require specific attention in future analyses, data presentation, and interpretation of embryonic mammary gland phenotypes. Regionalized responses of the ectoderm to a change of gene function may also provide more insight in what regulates the variation in number of mammary glands within and among species.

We have shown that several concepts about the ML and its role in MR formation need to be revisited. More insight in the temporal relationship between ML and MR formation, as well as directionality of the cell migration involved in these processes, will require further investigations. Studies using transgenic reporter mice that mark the ML by fluorescence, such as Krt17-GFP [85] and s-SHIP-GFP [80] are likely to provide valuable insights. However, time-lapse video-imaging has proven challenging so far, and optimization of culture conditions and imaging of 3D growth of embryonic flanks is required.

Certain aspects of morphogenesis, such as lumen formation and budding and branching morphogenesis, continue postnatally. Whether their mechanisms are similar during embryogenesis and postnatal life have not yet been rigorously examined. The embryonic MR is nonetheless a very practical model to study many such aspects of morphogenesis, and has the advantage of being less complex and relatively accessible compared to the postnatal mammary glands. Although many advances have been made in understanding the mechanisms that lead to morphogenesis of the embryonic mammary gland, there are still profound unanswered questions including the precise identity and tissues of origin of the earliest initiating signal(s), as well as the temporal and physical connections between the regulatory molecules identified to date.

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