

Chapter 2

Prenatal Mammary Gland Development in the Mouse: Research Models and Techniques for Its Study from Past to Present

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Abstract

Mammary gland development starts during prenatal life, when at designated positions along the ventrolateral boundary of the embryonic or fetal trunk, surface ectodermal cells coalesce to form primordia for mammary glands, instead of differentiating into epidermis. With the wealth of genetically engineered mice available as research models, our understanding of the prenatal phase of mammary development has recently greatly advanced. This understanding includes the recognition of molecular and mechanistic parallels between prenatal and postnatal mammary morphogenesis and even tumorigenesis, much of which can moreover be extrapolated to human. This makes the murine embryonic mammary gland a useful model for a myriad of questions pertaining to normal and pathological breast development. Hence, unless indicated otherwise, this review describes embryonic mammary gland development in mouse only, and lists mouse models that have been examined for defects in embryonic mammary development. Techniques that originated in the field of developmental biology, such as explant culture and tissue recombination, were adapted specifically to research on the embryonic mammary gland. Detailed protocols for these techniques have recently been published elsewhere. This review describes how the development and adaptation of these techniques moved the field forward from insights on (comparative) morphogenesis of the embryonic mammary gland to the understanding of tissue and molecular interactions and their regulation of morphogenesis and functional development of the embryonic mammary gland. It is here furthermore illustrated how generic molecular biology and biochemistry techniques can be combined with these older, developmental biology techniques, to address relevant research questions. As such, this review should provide a solid starting point for those wishing to familiarize themselves with this fascinating and important subdomain of mammary gland biology, and guide them in designing a relevant research strategy.

Key words Mouse embryo, Mammary gland development, Techniques, Mouse models, Explant cultures, Tissue recombination, Tissue interactions, Molecular interactions, Morphogenesis

1 Introduction

Already around 350 BCE Aristotle had documented that some but not all terrestrial and marine animal species have special milk-producing glands, usually with a teat or nipple as outlet, to which

the newborn can latch on for its feeding [1]. In some species he observed those glands only in females, in other species in both males and females [1], but even though males may lactate, e.g., in bats [2, 3] only females were observed to nurse the young. Perhaps this explains why these glands are called “mammary glands,” as a referral to the word “mama” or “mamma” for mother. Over 2000 years later, Linnaeus used the possession of mammary glands as the defining feature for a separate Class of animals, named Mammalia after the mammary gland [4].

Mammary glands are apocrine glands that reside on the ventral side of the trunk of adult mammals; most often they are present in pairs of which the singletons are displaced more or less symmetrically away from the ventral midline. In monotremes (platypus and echidna), each gland exists as one lobule budding off a single duct which is connected to a hair shaft [5]. Due to its small size, its milk producing capacity is low. Furthermore, in the absence of nipple or teat, the milk seeps out along the hair to be licked up by the newborn [6]. This type of gland and mode of excretion may reflect ancestral glands that birds used to moisten their eggs [5, 7], but it is relatively inefficient for nursing newborns. The low milk production per gland and wastage of milk is compensated by a high number (between 100 and 150 pairs) of glands in monotremes.

Mammary glands of marsupial (e.g., kangaroos) and placental (e.g., humans, whales) mammals have a large internal surface of secretory cells, owing to reiterated branching of the primary duct. Moreover, as all the milk of one gland drains to one teat or nipple from which the newborn can suckle, milk spillage is minimized. This generally ensures sufficient milk production per gland to feed one newborn. Compared to monotremes, marsupials and placentals can therefore do with fewer mammary glands. Indeed, their number of pairs of mammary glands ranges between 1 and 25 [8], in a correlation close to 1 for “average litter size” to “number of mammary gland pairs” across species [9]. With their maximum litter size seldom exceeding twice the average litter size, this ratio generally still leaves one gland available per newborn.

Interestingly, even if the number of mammary gland pairs is the same between some species, the location of these glands may differ between these species. For example, elephants, humans, and horses each have one pair of mammary glands, which is located at the chest in elephants and humans, but near the hind leg in the horse. This variation in position of the glands along the anteroposterior body axis seems to correspond to habitat, method of rearing, and degree of maturity of the offspring at birth [6].

Why would it be relevant to study the prenatal phase of mammary gland development? First of all, the mother’s milk is the only

source of nutrients for the newborn, and provides antibodies and other immune support as well until the newborn's own immune system becomes active [10–12]. Though humans may substitute their own breast milk by formula, most formula is still a dairy product. As such, mammary glands are directly crucial to the survival of mammalian species; and indirectly as well, through the close bond that nursing forges between the newborn and its mother. Even though the gland's milk-producing function is not required before adulthood, almost all aspects of mammary morphogenesis and functional differentiation already take place before birth. It is therefore not surprising that throughout the centuries, zoologists found the prenatal phase of mammary gland development important for study.

Moreover, almost all aspects of mammary morphogenesis and functional differentiation already take place before birth, only to be reiterated or enhanced postnatally under the influence of puberty and pregnancy hormones. Downstream of these hormones seem to act many of the signaling cascades that regulate prenatal mammary development [13–16]. Even stem cells, which are required to regenerate the mammary gland with each pregnancy, are already present in the prenatal gland [17–19]. As the prenatal mammary gland is relatively accessible for experimentation and is less complex in tissue composition than the adult mammary gland, it may be a practical additional or alternative research model for research questions pertaining to development of the postnatal mammary gland.

The regulation of the variation in number and position of the mammary glands raises additional interesting questions for developmental biologists about regulatory mechanisms creating this variation. For the high degree of similarity in shape and function between the multiple pairs of mammary glands in for example cats or pigs would suggest these glands are mere copies of each other. Yet the variation in number and position of glands between and even within species, and the heritable propensity for having too few or many mammary glands in for example sheep, pigs, humans, and macaques [20–26] indicates that each mammary gland must have some unique genetic component or protein activity that determines whether its development will be initiated and continued or not. Insights in these differences between the pairs and even between the left and right counterparts of each pair [27] may affect our thinking about the extrapolation of results obtained with one gland to other glands.

Of particular interest are the parallels in tissue interactions and molecular activity between prenatal mammogenesis and mammary tumorigenesis and metastasis [28–33]. Although better screening, care and treatment options for breast cancer have improved survival chances for patients with breast cancer over the past twenty years, this cancer is still the second leading

cancer-related cause of death for women worldwide [34, 35]. Progress in finding even better therapies is impeded by the wide heterogeneity in the molecular mechanisms of the wide variety of breast cancer types, only 2–10 % of which seems to have a familial component [36, 37]. As embryonic mammary glands are less complex and heterogeneous in tissue composition than adult mammary glands and tumors, and are easily accessible and available, new candidates for nonfamilial forms of breast cancer may be identified through the study of prenatal mammary gland development [28, 38].

For obvious reasons of ethics, human fetuses are insufficiently available for such studies. Comparative studies from the past have revealed that prenatal mammary gland development in rabbit embryos closely resembles that in human fetuses [39]. Nonetheless, currently most research on prenatal mammary glands is done in mice, and some of the techniques are optimized for use on this research model in particular, despite a few morphogenic differences in mammary development between men, rabbit, and mice [40, 41]. The choice for mice is largely based on the wealth of genetically engineered mice becoming available since 1989 [42]. Several of the genes that have so far been identified as regulators of early mammary gland development in the mouse embryo are known to also underlie defects in prenatal mammary development in humans [33, 43, 44]. Those findings validate the use of mouse embryos as a model for human prenatal mammary development.

Therefore, this review focuses primarily on mammary development in mouse embryos. It takes the approximate chronological order in which techniques were developed and used to study developmental biology, as a basis to describe how insights were gained in the different aspects of mammary gland development in mouse embryos.

2 Macroscopic and Microscopic Aspects of Prenatal Morphogenesis of the Mammary Gland in Mouse

From the mid-nineteenth century onwards there has been a steady stream of publications pertaining to embryonic mammary gland development in a broad variety of mammalian species. The earliest studies were based on macroscopic analysis of embryos to assess the number, positions, and external morphology of mammary glands, and microscopic analysis to study tissue composition and internal morphology of mammary glands at different embryonic ages.

Determination of embryonic age: For many species, embryos were obtained by chance without knowledge of the onset of pregnancy and age of the embryo. Size (e.g., crown–rump length) or weight measurements of embryos of different mothers were

used to assess the relative chronological age between embryos of different pregnancies. Although this is a helpful method in the absence of knowledge of the onset of pregnancy, size and weight are not precise determinants of (relative) chronological age, due to the normal variation in size and weight of embryos at any given developmental age.

Already in the early nineteenth century, rats, rabbits, and mice were kept in captivity for research purposes [45]. In captivity, the onset of pregnancy can be controlled. If the day–night (light–dark) cycle is kept regular, female mice in estrous will ovulate at around the middle of dark time, and produce more pheromones that entice the male to copulate. Copulation results in production of a sturdy white vaginal plug in the female that remains present for about half a day. Nowadays, in a laboratory setting, the middle of the dark time is often conveniently set to be around midnight. Therefore, noon of the day a vaginal plug is observed, is usually considered embryonic day 0.5 (E0.5), assuming copulation resulted in a pregnancy. The female is then separated from the male, and monitored for signs of pregnancy. Embryos are collected at the desired age for study. Embryos of the same pregnancy, thus same chronological age, will differ in their true developmental age. The relative developmental stage of embryos within one batch can be assessed by their progress in a developmental process that is particular for that chronological age, e.g., the number of somites between E8 and E12, and number of branches of the salivary gland at E13, unless one compares wild type embryos with littermates that carry a genetic mutation that disturbs the developmental process that is used for staging. Note that in the older literature, and occasionally in current publications, the progress of pregnancy is counted only in full days, and some may consider the day a plug is observed as embryonic day 0 (E0), while others consider it day 1 (E1). This may lead to small discrepancies in the literature regarding the timing of morphogenetic events of mammary morphogenesis. The different speeds of embryonic development between different mouse strains may be another source of small discrepancies in the literature regarding the timing of morphogenetic events.

Histology: Early descriptions of the murine mammary glands were based on microscopic analysis of histology, for which embryos were treated with a fixative, dehydrated, embedded in wax, and sliced into sections with a minimum thickness of 4 μm , and stained with a variety of chemical solutions to facilitate the recognition of different cell or tissue components (nucleus, cytoplasm, extracellular matrix fibers, etc.) [45–48]. From around the 1970s–1980s, histology was also performed on frozen sections, or specimens were embedded in a plastic or epoxy resin, to cut semi-thin (1 μm) sections which provide a higher resolution of intracellular structures [49, 50]. Such histological

studies led to most of the insights about morphogenesis as described further below.

Electron microscopy: From the 1970s onwards a few studies incorporated scanning electron microscopy of whole embryos to analyze changes on the surface of the embryo associated with mammary development [51–54].

Microscopy of whole glands: Embryonic skins can also be peeled off the embryo and mounted on a microscope slide for examination of gross morphology of the rudimentary glands under bright field stereoscopy. When the skin is peeled off sufficiently thin, transmitted light allows recognition of the rudimentary gland without further treatment of the specimen. After E16.5 the mouse epidermis becomes keratinized and subdermal fat develops. Visualization of the mammary rudiments (MRs) can then be enhanced by defatting and staining the skins with carmine alum [52, 53], according to a protocol routinely used for adult mammary glands [55].

3D-reconstruction of mammary rudiments: Recently, the application of bioinformatics and image analysis to digital images of histological preparations, or optical sectioning of intact fluorescently labeled MRs has allowed to generate 3D-constructions of complete series of (optical) serial sections through mammary rudiments [54, 56–58]. Different tissue components or differently labeled cell types can be identified manually or automatically, allowing measurements of volume, and proportions of different cell populations as well as recognition of regionalized distribution of specific cell populations within the MR [54].

2.1 Brief Overview of Macroscopic and Microscopic Aspects of Embryonic Morphogenesis

This section will only briefly describe the morphogenetic stages in mouse embryos, just to introduce the terminology and concepts of the field and facilitate the understanding of the subsequent passages of this current review. For more details on morphogenesis, the reader is referred to previously published reviews [41, 47, 53, 59].

Mammary gland development takes place along the ventrolateral boundaries in the surface ectoderm (i.e., the prospective epidermis) of the embryonic trunk. One could draw an imaginary line called *mammary line* or milk line (ML) extending from axilla (armpit) and inguen (groin) along both boundaries (Fig. 1). These boundaries are histologically detectable in the surface ectoderm as the junctions between squamous cells on the ventrum (belly) and cuboidal cells on both flanks.

In the course of the tenth day of mouse embryogenesis (E10.5), cuboidal cells along the two MLs first elongate to a columnar shape, rapidly followed by multilayering [45, 60, 61]. This cell elongation and multilayering occurs in three separate *mammary streaks* per ML: One extends between the forelimb and hindlimb and is approximately 30 cells wide, while separate streaks develop in the

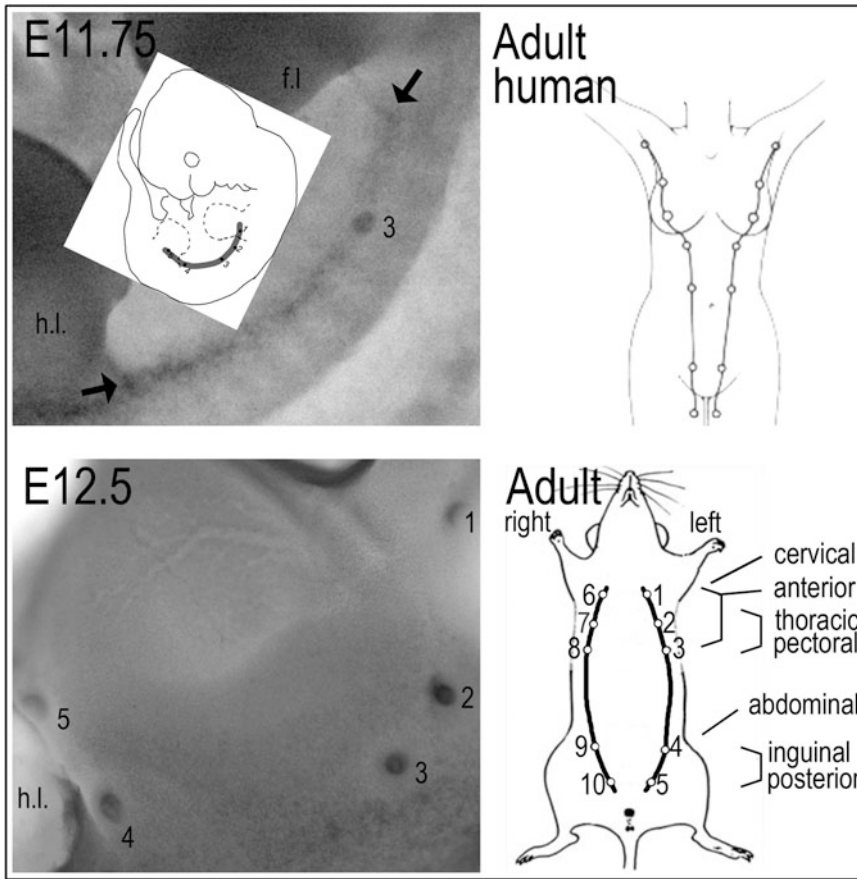


Fig. 1 Position of the mammary line and rudiments in embryo and adult. The *left two panels* show mouse embryos at E11.75 and E12.5, hybridized with a *Wnt10b* probe which visualizes the mammary line (between arrows) and rudiments (numbered). The *inset cartoon* shows how the mammary line extends from axilla, along the flank, to inguin. In adult mice and humans imaginary mammary lines can still be drawn more ventrally, connecting all sites where mammary glands reside, normally five pairs in mouse and one pair in human, but occasionally supernumerary mammary glands develop at other sites as indicated with *open circles*. In mouse, the embryonic mammary rudiments are usually numbered as pairs 1 through 5 in anteroposterior order, but in adult mice the glands may be indicated by individual number (1–10), or position on the trunk. *fl* forelimb, *hl* hindlimb. Adapted from ref. [27], with permission

axilla and inguen [61]. This marks the onset of mammogenesis. These streaks extend towards each other, and ultimately represent one continuous histologically detectable *mammary line* on each flank (Fig. 1). In species like rabbit, the MLs rapidly become elevated above the surface ectodermal landscape and are therefore called mammary ridges [41, 51]. At designated positions along the left and right ML, mammary glands will develop as symmetrically located pairs, of which the number varies in a species-dependent manner. They undergo a series of morphological changes or stages with each their own name as described below and depicted in Fig. 2.

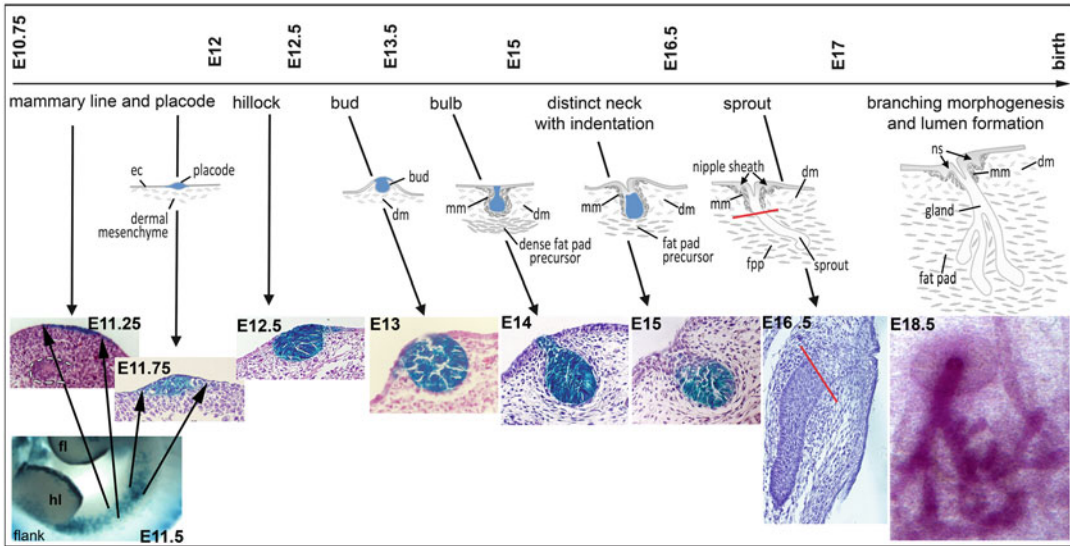


Fig. 2 Stages of mouse mammary morphogenesis in female embryos. Mammogenesis starts with the induction of mammary streaks which fuse into a continuous line from E10.75 onwards (shown as a lateral view on the flank of a TOPGAL-F stained E11.5 embryo) while at designated positions placodes are formed asynchronously before E12. Histological sections of TOPGAL-F stained embryos and cartoons with the blue TOPGAL-positive domains, illustrate how the epithelial mammary placodes transform to hillocks and spherical buds that are first raised above the landscape of surface ectoderm (ec), but by E13.5 they subside below the surface. By then, some mammary rudiments have acquired a bulb-shape, and a few layers of contiguous dermal mesenchyme (dm) condense around all buds/bulbs to become mammary mesenchyme (mm). By E14.5, subdermal mesenchyme differentiates into the dense fat pad precursor. Then the neck area of the mammary epithelium begins to differentiate and forms a funnel-shaped indentation as the future outlet of the milk canal. Around E16, the tip of the bud/bulb breaks through the mammary mesenchyme and invades the fat pad precursor, while a nipple sheath develops at the neck area. Within a day, branching morphogenesis and canalization occur, such that the mammary gland resembles a miniature mammary gland before birth, as shown with a carmine-red stained fragment of an E18.5 skin with gland. Adapted from refs. [41, 53], with permission

The embryonic mammary gland(s) may be called *mammary primordium* (primordia), *mammary anlage(n)*, or *mammary rudiment(s)* (MRs) in reference to any developmental stage or none in particular. They include the mammary epithelium (ME), mammary mesenchyme (MM), and fat pad (FP), as these tissues develop in an interdependent manner.

In mouse embryos, multilayering is advanced in one subdomain per mammary streak and at the subaxillary and suprainguinal junction of these streaks. Between E11 and E12, five pairs of lentil-shaped mammary *placodes* arise in the axillae (MR1), at a subaxillary position (MR2), at the level of the diaphragm (MR3), at a suprainguinal position (MR4), and in the inguinae (MR5). Intriguingly, they arise asynchronously, not in numerical order, and independently of each other [41, 52, 53, 61, 62]. By increased multilayering, each placode becomes a *hillock* within half a day,

slightly elevated in the ectodermal landscape [45]. Each hillock grows larger and changes shape into a spherical *bud* between E12 and E13, still elevated above the adjacent ectoderm [53]. During that day, the ML disappears as a histologically and molecularly detectable entity [60], but even at later stages the name *mammary line* may still be used to refer to the imaginary line that connects all mammary glands on one flank.

Subsequently the buds invaginate deeper into the underlying dermal mesenchyme, such that they are no longer elevated above the ectodermal landscape [45, 47, 53, 63]. From bud-stage, some MRs simply elongate (MR2, MR4, and MR5) while in MR1 and MR3, the proximal part which connects to the overlying ectoderm, takes on the shape of a narrow neck, such that these MRs each resemble a *bulb* (MR1 and MR3) [54]. Meanwhile, the contiguous layers of dermal mesenchyme condense concentrically and differentiate into a specialized fibroblastic mesenchyme called *dense* or *primary mammary mesenchyme* or simply *mammary mesenchyme* (MM) by E13.5 [64]. Between then and E15.5 the mesenchyme around the neck of the MRs in male embryos condenses. The spherical part of most/all MRs becomes disconnected from the epidermis and nipples fail to form in males [45, 47]. In most mammalian species including human such drastic sex-specific differences do not occur.

In E13.5 female mouse embryos the MRs remain intact and continue to grow, though slowly, over the next two days. Meanwhile, around E14.5, a subdermal layer of mesenchyme condenses and differentiates into the *secondary mammary mesenchyme* or *dense fat pad precursor* (FP) consisting of presumptive adipocytes, fibroblast, endothelial cells, nerve cells, and perhaps other cell types [65]. By E15.5 rapid proliferation of ME cells provides a growth spurt particularly at the distal end of each bulb [46], which elongates and breaks through its surrounding basal lamina and *primary mammary mesenchyme*. At that time, the position of the MR is outwardly visible as a funnel-shaped depression in the skin; the position of the future nipple [53]. By E16.5, the bulb has elongated further into a solid cord of epithelial cells. This so-called *sprout* penetrates the *fat pad precursor*, which has now a much lower cell density than at E14.5. While the *sprout* undergoes bifurcation [66] and side-branching by E17 [41], small internal cavities appear and join each other to generate a canal [66]. Meanwhile, the skin adjacent to the neck of the sprout differentiates into a *nipple* [45, 47, 67], which becomes the outlet for the milk canal. By E18.5, most MRs have undergone several rounds of reiterated branching and resemble a miniature *mammary ductal tree* by E18.5. MR2 and MR3 have the most branches, and MR5 may just show one bifurcation [53]. Birth is expected between E19.5 and E21.5 depending on the strain of

mice. By then, and in the context of differential growth speeds of different regions of the body, the imaginary mammary lines have acquired a more ventral position than at E11.5, and the MRs are spaced differently along those lines, such that MR1, MR2, and MR3 attain pectoral/thoracic positions, while MR4 and MR5 reach a low abdominal respectively inguinal position as seen in adults (Fig. 1).

2.2 Overinterpretation of Static Histological Data as if Tell-Tales of Kinetic Events

Although histological data only provide static information, they were in some cases used to make unfounded conclusions about kinetic events, such as the histological ontogeny of the ME and mechanisms of its morphogenesis. For example, Bresslau concluded that the ME must be of ectodermal origin, as in the several species he had examined so far, he had found no apparent boundary between the mammary placodes and the surface ectoderm, while these two tissues are separated from the underlying dermal mesenchyme by a continuous basement membrane [8]. Moreover, without measuring proliferative activity or pressures, Charles Turner and Elisio Gomez attributed the multilayering of the epithelium in the ML and MRs to rapid proliferation of the basal cell layer of these structures. They also considered the condensation of the underlying dermal mesenchyme a consequence of an increased pressure on the dermis by the multiple layers of epithelium [45]. Decades later, Albert Raynaud argued, equally without proof, that mesenchymal condensation was a result of local fluid extraction from the dermis by the ectoderm, which also led to enlargement (elongation) of cells in the mammary line [47].

However, Boris Balinsky challenged the presumed role of cell proliferation in multilayering as he observed too few mitotic cells (which he scored by the absence of a nuclear membrane) in the ML and MRs of E11–E14 mouse embryos to account for the rapid increase in ME cell number. He suggested that growth must be provided by surrounding ectodermal/epidermal cells streaming towards the places where the MRs are forming, but had no technique in place to demonstrate such cell movements [46, 68].

2.3 Scanning Electron Microscopy (SEM)

Propper used SEM to scan the surface of rabbit embryos at an age when their mammary ridge was clearly elevated. On the apex of the ridge, he observed occasional cells with a rounded cell body and filopodia-like extensions along the length of the ridge. He proposed these cells as “wandering” cells migrating towards sites of mammary placode formation [51]. At that time, it was assumed that the MLs are complete and continuous between axilla and inguen prior to placode formation, and that MRs will subsequently derive from the ML by localized enhanced cell proliferation ([45] and references therein). Interestingly, Propper had already called

for more nuanced thinking about that dogma, as the MRs in the axilla and inguen of rat and rabbit seemed to develop without apparent connection to the region of the mammary line on the flank between forelimb and hind limb ([69] and references therein). Nonetheless, his SEM data were extrapolated to mammogenesis in the mouse embryo, and the dogma now became that the MRs derive from the ML by cell migration, still implying the ML is complete before MR formation starts [60]. However, although Propper's SEM data may suggest cell migration, it is still static data, and no formal proof for migration. Moreover, contrary to rabbit embryos, mouse embryos do not form an elevated mammary ridge, and the ML in mouse embryos most likely only resembles the apex of the ridge in rabbit. In hindsight, the supposedly migratory cells at the apex of the ridge were detected at an embryonic age when the placodes are already present and transitioning to the hillock stage [41]. Thus one can also question the relevance of these supposedly migratory cells for the initiation of placode formation, as well as the validity of the extrapolation of the SEM data from rabbit to mouse.

Notably, like Bresslau, also Balinsky, Propper, and Sakakura assumed that the epithelial compartment of the MR is of pure ectodermal/epidermal origin. Nonetheless, one could for example also argue that dermal cells may locally traverse the basement membrane and contribute to the emerging mammary placodes, but that the sections may have been too thick, or not examined in sufficient numbers, to observe examples suggesting such events. It took another 45 years and development of tissue recombination techniques to unequivocally confirm the pure ectodermal origin of the mammary gland epithelium [70], see Subheading 4.4.

3 Combining Microscopy with Cell Labeling Techniques to Explore Whether Cell Migration Contributes to Mammary Placode Formation in the Surface Ectoderm

Meanwhile, the possible involvement of cell migration in early mammogenesis was studied more aptly by labeling cells in a defined region, and verifying their position after a certain period of time.

3.1 Charcoal Depositions

Alain Propper deposited charcoal on explanted flanks of rabbit embryos and cultured the flanks for several days before harvesting them and determining the location of the particles in histological sections of the flanks. Charcoal deposited *outside* the mammary ridge never ended up in the MRs, but charcoal deposited *on* the slopes of the mammary ridge around the time of placode formation, was incorporated in the MRs within 24–48 h

[39]. Although these experiments demonstrate the involvement of cell migration, the significance of these data for mammaryogenesis in mouse was not clear, as mouse embryos do not form an elevated mammary ridge. The ML in the mouse embryo more closely resembles the apex than the whole width of the mammary ridge. If one wants to extrapolate Proper's charcoal data to mouse, one has to consider the possibility that ectodermal cells flanking the ML in mouse embryos may also contribute to mammary placode formation.

3.2 *DiI-Injections*

DiI can be injected in embryonic flanks in explant cultures. When injected near the presumptive ML at E10.5, the labeled domain expands in the course of 3 days, suggesting that cell migration occurs in that time [71]. Shortcomings of this technique are that the precise location of the prospective ML is undetectable, and the relevant site of injection can only be estimated; cells are not labeled individually but as a cluster; the explant undergoes extensive growth in 3 days, which on the one hand leads to the loss of focal plane due to 3D growth and makes live or time-lapse recording of the culture impossible, and on the other hand allows for expansion of the labeled domain simply by cell proliferation and passing on of the dye to daughter cells. A comparison of start point and endpoint of a cluster of labeled cells does not differentiate between expansion of the domain by cell proliferation or migration.

3.3 *Labeled Markers of Cell Proliferation Offer Opportunities for Cell Tracing in Early Mammogenesis*

Balinsky's low count of mitotic cells in the ML and MRs [46] was confirmed by injecting pregnant female mice with tritiated thymidine (^3H -TdR) and analyzing the incorporation of ^3H -TdR in the skin and developing mammary tissues by autoradiography of histological sections of embryos that were harvested several hours after injection. No ^3H -TdR was incorporated in the ME of the embryonic MR3 when females were injected at different time points at the 13th day of pregnancy, indicating a proliferative arrest in this ME between E13 and E14. This was in stark contrast to the high ^3H -TdR incorporation, thus high proliferative activity, of cells in the adjacent ectoderm/epidermis and mesenchyme [13]. However, when embryos were harvested and analyzed 24 h after injection, the neck of MR3 contained labeled cells. As ^3H -TdR rapidly degrades when not incorporated in cells, these cells must have been labeled about a day earlier. Given that no ^3H -TdR was incorporated in the ME proper at the preceding day, these positive cells must represent cells that resided in the adjacent epidermis at their time of labeling, 24 h earlier. The authors also labeled and harvested embryos at E14.5, and observed a high proportion of positive cells in the ME, indicating a resumption of cell proliferation [13].

Somehow, the results of the previous study were later referred to as if ME undergoes a 24 h proliferative arrest between E12 and E13.5, even though this study did not include time points before E12, and not all MRs. Therefore Lee et al. elaborated on this study, widening the time range from E11.5 to E13.5 and including all MRs separately [54]. They replaced the ^3H -TdR by the thymidine analog BrdU—which also rapidly degrades if it is not incorporated in cells—and used immunohistochemistry to detect BrdU incorporation in histological sections of embryos harvested 2 or 24 h after injection of the mother. They found almost no BrdU positive cells in the epithelium of the ML and all MRs. Although they found small differences between the MRs, their overall data confirmed Balinsky's low counts of mitotic cells, thus little to no proliferative activity in the epithelium of the ML and MRs between E11.5 and E13.5 [54]. However, if embryos were harvested 24 h after injection of the mother, the embryonic ME contained a high number of BrdU-positive cells. Their number was too high to be explained by proliferation of the initially rare BrdU-labeled cells present at 2 h after labeling. As such, cell proliferation was excluded as a significant contributor of the initiation and growth of MR formation, while cell migration was identified as a major contributor to the initiation and early growth of the ME up to E13.5 [54].

The disadvantage of the ^3H -TdR or BrdU labeling technique is that cells are still not individually traced; it does not reveal the exact directionality (e.g., along the DV axis, along the AP axis/ML or centripetal aggregation) and distance of migration, nor does it distinguish between the peridermal and basal cell layers of the ectoderm/epidermis as putative contributors to the ME.

Regardless and importantly, the contention that the mammary placodes are (solely) derived from the ML [45, 65] was contested by these data, as the ML itself would contribute mostly unlabeled cells. Moreover, ME growth was mostly explained by the influx of labeled ectodermal cells [54].

4 Organ Explant Culture and Tissue Recombination Techniques Uncover Continuous Reciprocal Tissue Interactions That Drive the Induction and Morphogenesis of Embryonic Mammary Glands

4.1 *Ex Vivo* Explant Cultures

In order to facilitate the manipulation of mammary gland development and to address questions concerning regulatory mechanisms of mammary development, an existing *in vitro* organ explant culture technique [72] was modified to support the growth of embryonic mammary glands *ex vivo*. With this purpose, Margaret Hardy cut out the ventral and lateral body wall including the ML region

of E10, E12, and E13 mouse embryos. She cultured them in adult cock plasma and chicken embryo extract in a watch-glass [73]. Boris Balinsky reduced the explants to a smaller strip of tissue encompassing the ML region, modified the medium, and also tried to culture explants of E8 and E9 embryos [68]. While these younger explants necrotized, explants from E10 and older embryos survived in both Hardy's and Balinsky's experiments. These were examined directly under the stereoscope, or prepared for sectioning and histological analysis. Both Hardy and Balinsky observed MRs in a small percentage of cultured E10 embryos, even though these embryos had no MRs at the time of explantation. Later also Etienne Lasfargues and Margaret Murray [74] successfully grew mammary glands in explants of E10 embryos. While explants of E10 embryos yielded MRs at different stages of morphogenesis within the same E10 explant after 18 days of culture, MR development was more successful and at a more consistent speed in E12 and E13 explants. In such explants, development was only slightly delayed to in vivo development and even progressed to branching morphogenesis [73].

4.2 Tissue Recombination

More than a decade later, Alain Propper and coworkers successfully modified the culture technique for rabbit embryos, albeit that the explants did not attain branching morphogenesis [75]. At the time, developmental biologists were discovering important roles for mesenchymal tissues in organ development. In that context, Propper wanted to assess whether MR formation is an intrinsic property of the ectoderm, the mesoderm, or induced in the ectoderm by the mesoderm. He dissected embryonic flanks, separated the mesenchyme from the ectoderm/epidermis by a mild trypsin digestion, and put them in culture. The mesenchyme or epidermis alone did not give rise to MRs, and often degenerated. He also separated the mesenchyme and epidermis from the head region, and then recombined flank mesenchyme with head epidermis and vice versa (the so-called heterotopic tissue recombinations). Head mesenchyme did not induce a ML or MRs in E12 flank epithelium, although it would sustain MRs present in E13 and E14 flank epithelium. By contrast, flank mesenchyme from E12 (no mammary line/ridge yet) to E14 (hillock stage) embryos did induce a mammary ridge and subsequently MRs in head epithelium [76, 77]. Propper went on to recombine flank mesenchyme of E12 (pre-ML) or E13 (ML) rabbit embryos with chick or duck epidermis just prior to (E6, E7) or after (E8, E9) feather bud induction, and even with chick amnion or chorion (the so-called heterospecific recombinations). In all cases he observed spherical buds resembling mammary buds. In recombinants with bird epidermis he observed concentrically condensed mesoderm around these buds, and upon longer culture periods, these buds developed deep invaginations with a lumen, thus morphologically closely resembling mammary sprouts [78,

79]. These experiments showed that the initiation of mammogenesis is not intrinsic to the ectoderm/epidermis, but induced by local factors in the flank mesenchyme underlying the ML in rabbit embryos. Moreover, the flank mesenchyme exerts an *inductive* role, and can even induce mammary morphogenesis in epithelium that normally does not form mammary glands, even from other species as long as this epithelium is not yet committed to a particular fate. Similar heterotopic and heterospecific experiments at slightly different embryonic ages revealed that once the ML is formed, it needs mesenchyme for its fractionation into MRs. However, this mesenchyme need not be the flank mesenchyme, thus any mesenchyme can take over this *permissive* role.

While Propper was working on the rabbit, Klaus Kratochwil aimed to improve morphogenesis of mouse embryonic mammary glands in culture. He replaced the watch-glass used by Hardy, Balinsky, and Lasfargues and Murray with Grobstein's special glass organ culture dishes [80] that have a central depression containing 0.7–0.9 ml of nutrient medium. He placed a thin ($22 \pm 3 \mu\text{m}$) filter with an average pore size of $0.35 \mu\text{m}$ on the depression such that it was in contact with, but not submerged in the medium. At the air–liquid interface on these filters, he cultured either intact MRs with a fair amount of subjacent mesenchyme and a small piece of epidermis, or he separated the ME from its subjacent mesenchyme and cultured the two tissues in isolation or recombined them with each other [81]. With these techniques, he was able to achieve normal mammary morphogenesis in organ culture, including the formation of a nipple with nipple sheath, a ramifying ductal system based on monopodial branching as is typical for mammary glands, and adipose tissue. However, when he recombined E12 and E16 ME with E13 salivary mesenchyme, he observed a dichotomous branching pattern that is typical for a salivary instead of mammary gland. From his experiments, he concluded that ME requires any mesenchyme to continue growth and morphogenesis; that the organ-specific morphology is induced by the mesenchyme; and that at E16, the ME is not yet committed to this mammary-specific morphology [82].

4.3 Applications of the Explant Culture Technique

Kratochwil used his culture technique mostly for recombinant explants to study aspects of the sexual dimorphism of mammary development observed in mouse, as described below. But even nowadays, the technique of culturing explants in the air–liquid is still frequently used with individual MRs, tissue recombinants, or whole flanks. It is very amenable to the introduction of experimental variables that also address fundamental questions about the nature and role of tissue–interactions in organ development in a very precise and elegant manner, as will become clear in the course of this review. It is a practical method to monitor daily progress of mammary development, especially in cases when for example a

prenatally lethal mutation would prevent mammary development *in vivo*. It facilitates the study of the roles of genes or proteins of interest in tissue-interactions by electroporation of expression constructs [71, 83], creating heterogenic (female/male or wt/mutant) tissue recombinants at developmental stages of interest [84], or by manipulating the levels of soluble proteins by adding them to the medium or implanting slow-release beads coated with proteins in flanks in culture [57, 60, 62, 71, 85]. A detailed protocol for dissection of flanks and individual MRs and tissues has been published recently [29, 56, 58] and is illustrated with movies as well [86]. Even if in the latter protocol, tissues were treated with RNALater™ or a fixative for gene expression or protein analysis, the general steps of dissection are similar for cases where tissues are harvested for culture. Additional protocols describe variations on the culture protocol to analyze branching morphogenesis or perform tissue recombination [83, 87–89].

4.4 Transplantation or Grafting of Explant Cultures

One drawback of the *in vitro* explant culture technique is that the medium needs to be daily replaced, and does not contain the maternally derived or self-produced hormones that may circulate through the bloodstream of mammalian embryos. To test the morphogenic effect of pregnancy hormones on embryonic MRs, Teruyo Sakakura and colleagues repeated Kratochwil's recombination experiments of E16 ME with E13 salivary mesenchyme, but subsequently grafted the recombinants under the kidney capsule of syngeneic female mice, which were then made pregnant. Similar to Kratochwil, Sakakura observed a salivary gland morphology in her transplanted recombinants, and in addition she found that this epithelium produced milk proteins. Thus, morphological development and functional differentiation of the ME are not coupled, and commitment to the lineage-specific differentiation program is established in the ME before E16 [90].

Two decades earlier, K.B. DeOme and colleagues had published the successful grafting and growth of ME of an adult donor mouse into the mammary fat pads of 3-week-old female mice that was cleared of its own mammary epithelium [91]. After a desired period of growth of such grafts, the fat pads are dissected, fixed, dehydrated, defatted, and stained with hematoxylin/eosin or carmine alum for stereoscopic analysis of the outgrowth [55]. As the mammary fat pad is the natural environment for ME from around E16 onwards, Sakakura next tried if E16 embryonic ME could also thrive in such cleared prepubertal fat pads. Indeed this was the case, and even MRs from E13 donors developed rigorously and with normal branching patterns in such cleared fat pads [92]. She observed that the fat pad also sustains the growth of embryonic primary (dense) mammary mesenchyme (MM) and secondary mammary mesenchyme or fat pad precursor (FP) and studied

their effect on adult ME by not clearing the host fat pad prior to grafting. She identified different effects on adult ME morphogenesis: Where adult ME was in contact with MM, it underwent hyperplastic branching in a monopodial pattern without ductal elongation, whereas adult ME in contact with FP underwent monopodial branching and ductal elongation, and as such was indistinguishable from a normal adult gland [92]. She observed a close resemblance between the MM-induced hyperplastic nodules and hyperplastic nodules that were already at the time considered preneoplastic lesions [91, 93], and recognized that it was of importance to study whether the MM has a tumor-enhancing potential and if so, how this potential was suppressed in the embryo [30, 92].

Building on the works of Kratochwil and Sakakura, and with a similar interest in the role of mesenchyme in organ development, Cunha and coworkers combined recombined E13 mouse mesenchyme underlying the ML with E13 rat ectoderm from the dorsal or ventral region (thus not from the ML) and transplanted these heterospecific, heterotypic recombinants in lactating female mice. The developing ME in such recombinants was entirely rat-derived, finally confirming the ectodermal origin of mammary gland epithelium [70] as suggested decades earlier by Bresslau [8], Turner and Gomez [45] and Balinsky [68].

Currently, the technique of transplanting embryonic mammary tissues in the cleared prepubertal fat pad is still used regularly, e.g., when embryos of mutant mice do not survive long enough to monitor mammary development, or to test whether observed mammary phenotypes in mutant embryos are due to the altered gene function in the ME, in the MM or in the FP [14, 52, 94–97].

5 Techniques to Study the Role of Steroid Hormones in Prenatal Sexual Dimorphism of Mammogenesis

Observations of sexual dimorphism.

5.1 Histological Analysis

In 1933, Turner and Gomez already mentioned that in male mouse (and rat) embryos, contrary to other species they knew, the MRs become detached from the epidermis and do not form nipples [45]. Albert Raynaud studied this in more detail and observed no notable differences in MRs between male and female embryos of E12 to E14 [98] and Raynaud (1947) cited in ref. [47] though Kratochwil observed a slightly smaller size of MRs in E14 males compared to females [63]. At E15, the MM around the neck of the bud/bulb is in males much more condensed than in females and pyknotic cells are present in the neck epithelium at E15. Soon the bulb of the MR detaches from the epidermis, likely due to this

mesenchymal constriction and epithelial cell death ([47] and Raynaud (1947) cited therein). Notably, not all five pairs of MRs in males undergo this process: Raynaud observed that the fifth pair of MRs apparently regresses without prior separation from the epidermis [99] and considerable variations were observed between strains [63, 100].

5.2 Manipulation of Embryonic Mammary Development In Utero

Albert Raynaud and Marcel Frilley hypothesized that the differences in mammary development between male and female embryos may be due to functional differentiation of the gonads occurring before that time. To test this, they performed a fetal gonadectomy by X-ray irradiation of the gonads of E13 mouse fetuses of both sexes in utero, which they then allowed to develop in utero until E18.5. In both gonadectomized sexes, the MRs developed as in untreated female embryos, indicating that by default, mammary development proceeds along a female program, which does not require embryonic gonadal function. The perturbed mammary development in males is due to gonadal function in male embryos (Raynaud and Frilley (1947, 1949), cited in ref. [47]).

Unraveling the actions of testosterone.

In other experiments, pregnant females were injected with male steroid hormones. This led to involution of the MRs in female as well as in male embryos (Raynaud (1947a, 1949) cited in ref. [47]; [101]), whereas injection of a synthetic antiandrogenic steroid prevented the regression of MRs in male embryos [102]. Together, these experimental data demonstrated that the MRs need no embryonic gonadal secretions for their development, and that the embryonic testes are responsible for perturbed mammary and nipple development in male embryos [47].

5.3 Explant Culture and Organ Coculture

Kratochwil argued that gonadectomy may affect other endocrine organs in the embryo, and the injections may create a hormonal imbalance in the pregnant mother. Therefore, the abovementioned experiments could not answer the question whether the steroid hormones act directly or indirectly on the MRs, whereas explant culture experiments could. He observed a female developmental program in E12 and E13 mammary explants of both male and female embryos. However, of E14 male explants, MR2, MR3, and M5 were very susceptible to regression, while MRs that survived (50 % of MR1 and MR4 and some MR2 and MR3), resumed growth along a female developmental program albeit with a 2-day delay. In explants of E12–E15 females that were cocultured with E13 testes, all E12–E14 MRs regressed, while 75 % of the E15 MRs survived. Kratochwil obtained similar results when he replaced the testes by testosterone. He therefore concluded that testosterone acts directly on MRs, without necessary involvement of other endocrine organs, although the speed and

nature of the morphological response to testosterone may differ between MR pairs. Moreover, the arrest or degeneration of MRs as observed in males is not dependent on the genetic sex of the MRs proper, but on the presence of androgenic hormones in the embryo. Importantly, these androgenic hormones can exert their effect only during the limited time-window between E13 and E14 [50, 63].

5.4 Analysis of (Spontaneous) Mutant Mice

At the time, Lyon and Hawkes had just recovered spontaneous mutant mice carrying an X-linked mutation, X^{Tfm} , leading to testicular feminization [103] that was attributed to a nonfunctional androgen receptor [104, 105]. Kratochwil and Schwartz used these mice to uncover whether the androgen response of male MRs occurs in the ME, MM, or both [106]. They made heterogenic (wild type/mutant) recombinations of ME and mesenchyme of male embryos only and cultured them ex vivo in the presence of testosterone. While all recombinants with mutant mesenchyme underwent female morphogenesis, approximately 60% of the recombinants with wild type mesenchyme underwent the typical developmental arrest or regression normally seen in wild type males. Kratochwil and Schwartz therefore concluded that in male embryos, testosterone only acts on the MM and not the ME, and that the observed epithelial cell death in male MRs is mediated by the mesenchyme.

5.5 Radioactive Cell Labeling and Autoradiography

To test whether cell proliferation contributed to the higher density of MM compared to dermal mesenchyme, Kratochwil and colleagues cultured explants several hours in the presence of tritiated thymidine to label cells in S-phase prior to harvesting the explants for histology combined with autoradiography. The virtual absence of radioactivity in the MM indicated that the higher density of this mesenchyme compared to the dermal mesenchyme is not due to increased proliferation [50]. Later they immersed skin strips with mammary glands from freshly dissected embryos in radiolabeled testosterone, and processed them for histological sectioning and autoradiography or for radioactivity measurements in tissue extracts [107, 108]. They such established that the greatest testosterone-binding capacity is localized in the dense MM adjacent to the ME.

5.6 Heterogenic wt/ Mutant Explant Cultures

Because the higher cell density of the MM could not be attributed to locally enhanced proliferation [50] Kratochwil and colleagues wanted to investigate whether mesenchymal cell migration towards the bud contributes to the condensation of the MM. They made heterogenic with *wt* ME with adhering MM and a large mass of X^{Tfm}/Y MM and dermal mesenchyme, and vice versa. In this case, all recombinants of wt epithelium and adhering MM responded to testosterone despite their environment of

androgen-insensitive *Tfm* mesenchyme, whereas recombinants of *Tfm* epithelium and MM with a mass of wt mesenchyme showed no androgen response. Thus it seemed that the mesenchymal response is initiated at the epithelial–mesenchymal interface only, and does not involve migration of distant mesenchymal cells toward the ME. This was further supported by experiments with recombinants of wt epithelium with X^{Tfm}/X mesenchyme, i.e., from heterozygous females, instead of from mutant males. In cells of females at an early embryonic age, one of both X-chromosomes is randomly inactivated and remains inactive in daughter cells. This random X-inactivation resulted in clusters of androgen-responsive cells with an active wt *X* chromosome, and clusters of androgen-insensitive cells with an active X^{Tfm} chromosome in the MM of X^{Tfm}/X females. The mesenchyme of such recombinants cultured in the presence of testosterone showed similar clusters of mesenchymal condensation representing clones of cells with an active *X* chromosome, and clusters of loose mesenchyme represented clones of cells with an active X^{Tfm} chromosome. This heterogeneity also indicated that condensation, once initiated, does not spread across the mesenchyme independently of the hormone [49, 50].

Recombinants of wt male MM with wt epithelia of other organs did not show this androgen response, indicating that an interaction with specifically the ME is required for the mesenchyme to pack densely in response to testosterone. Moreover, wt ME induces this testosterone response even in wt mesenchyme that is normally not in contact with ME, e.g., the mesenchyme that is situated in between positions where MRs form along the ML [49]. This was later attributed to the localized induction of a testosterone-binding capacity by the ME in the adjacent mesenchyme [107, 108], provided by androgen receptors [109]. In addition, heterochronic recombinants (different in age) of wt ME and mesenchyme showed that the developmental age of the MM, but not of the epithelium is key to this response [50].

Notably, this strong androgen-response in males is specific for rats and mice, as in other species under study, mammary gland development in male embryos proceeds the same as that in females embryos. In correspondence, testosterone binding was not observed in MM of rabbit embryos, and heterospecific recombinants of mouse ME with rabbit mesenchyme did not exhibit any condensation in response to testosterone [49, 108].

5.7 Androgen Receptor Activation in Females In Utero

However, low concentrations of testosterone have been found in female mouse embryos [110], and the MM of female mouse embryos also expresses androgen receptors [109]. While androgen receptor activation was long considered to be nonexistent or too low in females to affect their mammary development, E18 females

with an intrauterine position in between two males (2 M females) have smaller mammary glands than females flank by two females (0 M females), which is likely attributable to androgen receptor activation in 2 M females by testosterone diffusing from their flanking males [111].

Unraveling the actions of estrogens.

Mammary development in gonadectomized male and female embryos proceeds as in normal female embryos. Although Raynaud therefore concluded that MRs need no embryonic ovarian secretions for their development (Raynaud and Frilley (1949), cited in ref. [47]), he did nonetheless consider the possibility that maternal hormones may be present in the amniotic fluid or traverse the placental barrier, and as such may contribute to the default, female, developmental program for MRs (Raynaud (1947), cited in ref. [47]).

**5.8 In Utero
Manipulation of Mouse
Embryos**

Indeed, MRs are able to respond to estrogenic compounds, as the injection of high doses of estrogenic compounds in pregnant females stimulated nipple development [112, 113] and led to failure of the sprout to elongate and branch [114–120]. When 16-day pregnant females were subcutaneously injected with the radiolabeled estrogenic compound diethylstilbestrol, followed several hours later by dissection and cryosectioning of the embryos for histology and autoradiography, these estrogens were traced back in the nuclei of E16 MM, but not ME [121]. This location corresponded nicely with the aforementioned phenotypes caused by exposure to high levels of estrogenic compounds.

**5.9 Gene and Protein
Expression Analysis**

Meanwhile, molecular cloning techniques had resulted in the identification of two (α and β) nuclear estrogen receptors (ERs), with different activation responses to different estrogenic compounds. In situ hybridization of sectioned embryos with mRNA probes for these genes demonstrated that both genes were expressed in the MM of E12.5–E14.5 (other ages not tested) mouse embryos, with higher levels of ER- α [122, 123]. Transcripts of both genes were also detected in the E18 MR, while only ER- α is expressed at immunohistochemically detectable levels in the fat pad precursor [111]. Extracts of E12.5, E14.5, and E16.5 male and female mouse embryos activate ER- α —though not ER- β —in vitro, indicating that estrogens do naturally circulate in embryos of both sexes [124]. It is conceivable that these estrogens may activate the ERs in the MM. Progesterone receptor expression has been detected in the E14.5 ME, but whether it is functional has not been assessed [125].

As mentioned above, embryonic MR development does respond to treatment of the mother with normal or synthetic

estrogens [63, 115–120], and more recently, the xeno-estrogen and endocrine disruptor bisphenol-A, a phenol-derivative that leaks from most plastics, has been demonstrated to affect embryonic MR development in mice as well [111]. Although each of these studies describes different effects—which may be due to differences in the timing and length of exposure and chemical structures used—together they certainly underscore the sensitivity of embryonic mammary development to ER-activation. Even in the absence of a significant role for endogenous ER-signaling in normal embryonic mammary development, this sensitivity to xeno-estrogens is highly relevant for further study, as exposing pregnant female animals (e.g., in agriculture) and humans to estrogenic compounds may lead to serious malformations of the mammary gland and nipple in the embryos, and thus to functional insufficiency in postnatal life [47]. Furthermore, inappropriate ER signaling in the embryonic MR may well predispose the mammary gland to cancer in postnatal life [126–130].

6 Models and Methods to study the Molecular Regulation of Embryonic Mammary Development

6.1 *In Situ Detection of Protein (Activity) and RNA Molecules*

Since the 1950s, studies on mammary gland development include questions pertaining to the activity and regulatory roles of molecules. For example, Balinsky [68] and Propper [131] observed fluctuating levels of alkaline phosphatase activity and RNA content in the ME and MM of the developing MRs of sectioned embryos, but could only speculate about the implications of these molecules and their fluctuations. When techniques for protein purification, antibody production and labeling also became available, they were first used to localize for example matrix molecules such as tenascin-C, laminin, and fibronectin, as well as milk proteins in histological tissue preparations of MRs [70, 132–134], soon followed by a plethora of other proteins. More recently, techniques to assay protein expression in preparations of whole mount MRs [56] and 3D-reconstructions of stained histological or optical sections of MRs were developed [54, 57, 58]. Meanwhile, techniques were also developed to synthesize labeled RNA probes, which are used to study gene expression patterns by whole mount in situ hybridization of whole embryos up to E13/E14, or by in situ hybridization of sectioned embryos of any age [52, 135–137].

6.2 *Spontaneous Mutant Mouse Models*

Almost four decades lapsed between Raynaud's discovery of hormonal control of mammary gland development [112, 113, 116–120, 138] and the identification of another molecular regulator of

mammary development. This began with the observation of absent MRs in E13 embryos of the spontaneous mouse mutant *Extratoes* (*Xt*) [139], but it took until 1993 until this mutation was identified as a functional null allele of the transcription factor Gli3 [140]. Other spontaneous mutations leading to mammary defects are the *X-linked testicular feminization* (*X^{lfm}*) encoding a dysfunctional androgen receptor [106], *Scaramanga* (*Ska*) representing a mis-regulated allele encoding the soluble factor neuregulin 3 (Nrg3) [141–143], *Tabby* encoding a functional null allele for the soluble protein ectodysplasinA1 (EdaA1) [85, 144], and *Spotch* encoding a functionally null Pax3 transcription factor [60].

6.3 Genetically Engineered Mouse models

Meanwhile, gene targeting techniques to generate genetically engineered mice (GEMs) [42] became widely used, and produced a myriad of constitutive, tissue-specific, and inducible mutant mice, in which the endogenous gene no longer produces a functional transcript of protein (knockout), or carries a domain deletion or point mutations that alters protein properties such as localization, binding affinity or enzymatic activity. In addition, transgenic mice were produced that carry exogenous DNA encoding a normal or mutant gene to increase expression levels of normal protein or produce high quantities of mutant protein, which outcompetes the normal. Most models studied for embryonic mammary gland development (Table 1) are constitutive knockouts and tissue-specific transgenic mice in which the promoter of either cytokeratin5 (Krt5) or cytokeratin 14 (Krt14) generates a functional null deletion or drives transgenic overexpression in the ectoderm/epidermis and the epithelial compartment of epidermal appendages such as teeth, hairs and mammary glands. In some cases these mutations are combined with lacZ or fluorescent (GFP) reporters that either mark the mammary line or rudiments (e.g., TOPGAL, s-Ship-GFP) or replace the expression of the endogenous gene (e.g., *Sostdc1^{LacZ}*) (Table 2).

The observation of a mammary defect in mutant embryos is usually accompanied by an analysis of the expression pattern of the normal gene in wild type (wt) embryos. This leads to an expansion of a database of suitable expression markers for the mammary tissues at various stages, as well as to hypotheses about the relevance of specific aspects of the spatiotemporal expression pattern for mammogenesis. Similarities in expression patterns of two genes in wt mice respectively in mammary defects in mutants of these genes may lead to additional hypotheses about epistatic interactions between these genes. Most of these hypotheses are tested ex vivo with explant assays, or in vivo by combining several mutations in one mouse to determine if one mutation restores or alters the mammary phenotype caused by the other mutation. During the past 25 years and especially since the beginning of this century, this has led to many insights in the molecular regulation of various stages of embryonic mammary gland development. Most of these

Table 1
Genetically engineered mice studied for embryonic mammary development

mouse genotype	description of allelic mutation				defect observed at stage:					remarks	references										
	spontaneous	functional null	ENU-induced LOF	targeted	From induction to hillock stage																
	hypomorphic allele	constitutive	tissue-specific	inducible	null	hypomorphic	loss of domain	ectopic expression	LatZ knock-in	MR1	MR2	MR3	MR4	MR5	super-numpy	bud to bulb stage	sexual dimorphism	sprout stage	nipple stage	branching stage	
wild type										●	●	●	●	●							
Single gene mutations	<i>p63^{-/-}</i>		*		*					○	○									[145, 146]	
	<i>Krt14-Dkk1</i> (loss of Wnt-signalling)			*	*		*			○	○	○	○	○						[147]	
	<i>Krt5-tTA;tetO-Dkk1</i> (loss of Wnt signalling)			*	*		*			○	○	○	○	○						[148]	
	<i>Tbx3^{tm1.1Rb/mmlPa}</i>		*	*	*	*	*			○	●/○	○	○	○	*					v.p.	
	<i>Fgf2b^{-/-}</i>		*	*	*	*	*			○	○	○	○	●	*					[149, 150]	
	<i>CMV-Cre;Rosa26-rtTA^{lox};tetO-sFgf2b</i> (dom.neg.)		*	*	*	*	*			○	○	○	○	●	○					[151]	
	<i>Krt14-Cre;Gata3^{lox/lox}</i>		*	*	*	*	*			●/○	○	○	○	○	○					[152]	
	<i>Gli3^{K1-1Rb/+} or Gli3^{X1/X1}</i> (extra-toes)	*								○	○	○	○	○	○	*				[54, 60, 139, 153, 154]	
	<i>Tbx2^{tm1.1Rb/mmlPa}</i>		*	*	*	*	*			○	○	○	○	○	○	*					[150]
	<i>Tbx3^{tm1.1Rb/+}</i>		*	*	*	*	*			○	○	○	○	○	○	*					[150]
Single gene mutations	<i>Nrg3^{Shy}</i> (scaramanga)									○	○	○	○	○	○					[141-143]	
	<i>Pax3^{ScpSp}</i> (splotch)	*								○	○	○	○	○	○					[60]	
	<i>Pax3^{g2/Hz}</i>	*		*	*	*	*	*		○	○	○	○	○	○					[60]	
	<i>Krt14-tTA;tetO-Wise</i>		*	*	*	*	*	*		○	○	○	○	○	○	*				[155]	
	<i>Lef1^{-/-}</i>		*	*	*	*	*	*		○	○	○	○	○	○					[135, 156]	
	<i>Pygo2^{-/-}</i>		*	*	*	*	*	*		○	○	○	○	○	○					[157]	
	<i>Krt14-Cre;Pygo2^{lox/lox}</i>		*	*	*	*	*	*		○	○	○	○	○	○					[157]	
	<i>TCF-tTA;tetO-Wise</i>			*	*	*	*	*		○	○	○	○	○	○			MR4 develops normal		[155]	
	<i>Krt14-Cre;β-catenin^{lox/lox}</i>			*	*	*	*	*		○	○	○	○	○	○					[155]	
	<i>Lrp4^{mdg/mdg}</i>	*								○	○	○	○	○	○	*				[155]	

Table 2
Reporter mice used in studies of embryonic mammary development

Reporter mice	Marks	References
TOPGAL-F <i>Egfl0⁻;Topgal-F</i> <i>Egfr2b⁻;Topgal-F</i> <i>Gli3^{Xt-1};Topgal-F</i> <i>Lrp4^{mdig};TOPGAL-F</i> <i>Sostdc1^{LacZ};TOPGAL-F</i> <i>Wise⁻;TOPGAL-F</i> <i>Nrg3^{Ska};TOPGAL-F</i>	Wnt signalling in epithelium; ML, MR	[148, 179] [60] [60] [60, 153] [155] [160] [155] [180]
TOPGAL-C <i>Lcf1⁻;TOPGAL-C</i> <i>Krt14-PTHrP;TOPGAL-C</i> <i>Dermo-Cre;β-catenin^{flax/flax};TOPGAL-C</i>	Wnt signalling in epithelium and mesenchyme	[181] [156] [166] [166]
BATGAL <i>Lrp5⁻;BATGAL</i> <i>Lrp6⁻;BATGAL</i> <i>Pygo2⁻;BATGAL</i> <i>Sostdc1^{LacZ};BATGAL</i>	Wnt signalling in epithelium and mesenchyme	[182] [161] [162] [157] [160]
Conductin^{lac/+} (=Axin2^{lac/+}) <i>Axin2^{CrtERT2/+};R26R^{LacZ/+}</i> <i>Axin2^{CrtERT2/+};R26R^{LacZ/+}</i> <i>Gli3^{Xt-1};Conductin^{LacZ}</i>	Wnt signalling in epithelium and mesenchyme	[155] [183] [183] [154]
TCF/LEF:H2B-GFP <i>Lrp4^{mdig};TCF/LEF:H2B-GFP</i>	Wnt signalling, similar to TOPGAL-F	[155, 184] [155]
Eda^{REP} <i>Edatm;Eda^{ZREP}</i> <i>Krt14-Eda;Eda^{LacZREP}</i>	Eda signalling	[175] [175] [175]
Krt17-GFP <i>Sostdc1^{LacZ};Krt17-GFP</i>	<i>Krt17</i> expression; epidermis	[160, 185] [160]
s-Ship-GFP <i>Nrg3^{Ska};s-Ship-GFP</i>	<i>Ship1</i> expression; ML	[186] [41] [180]
<i>Krt14cre;R26^{-floxedstop-LacZ}</i>	Cre, LacZ specifically in MRs from E12 onwards	[155]
<i>Krt14-tTA:tetO-Wise-GFP</i>	transgenic <i>Wise</i> expression in MRs from E12 onwards	[155]
<i>Msx1-LacZ</i>		[174]
<i>Msx2-LacZ</i>	transgenic <i>Msx2</i> expression	[174]
<i>BMP4-LacZneo</i>	transgenic <i>BMP4</i> expression	[164, 187]
<i>TrkB^{GFP/+}</i>	neurons	[167, 188]
<i>Lrp4-LacZ</i>	<i>Lrp4</i> promoter activity	[155]
<i>Wise-LacZ</i>	<i>Wise</i> promoter activity	[155]

This table lists all reporter mice, and their combination with gene mutations causing an embryonic mammary phenotype, known to date (early 2014)

insights have recently been comprehensively reviewed elsewhere [32, 33, 43, 44, 189–191]. Below, the focus lies on the experimental approaches that led to some of these insights.

7 Molecular Regulation of Patterning of the MRs in the Surface Ectoderm

From their tissue recombination experiments Propper, Kratochwil, and Cunha and Hom had concluded that the differentiation of ectoderm into mammary epithelium is induced by (then unknown) mesodermal/mesenchymal factors [49, 50, 69, 70, 76–79, 81, 82, 192]. Correspondingly, some GEMS with defective mammary induction (Table 1) carry a mutated version of a gene which in wt is among others expressed in the dermal mesenchyme at the time of ML and MR induction, e.g., the growth factor *Nrg3^{ska}*, and transcription factors *Tbx2* and *Tbx3* [28, 33, 149, 159, 193]. However, most GEMS with a known induction defect lack a gene that in wt is expressed in the somites, i.e., the mesodermal structures that give rise to vertebrae, ribs, muscles, and the dermal mesenchyme. These genes encode the transcription factors *Gli3*, *Pax3*, *Tbx2*, *Tbx3*, and likely *Hoxc6*, the growth factor *FGF10*, or retinoic acid receptors [32, 43, 44, 158, 189, 191, 194, 195]. This somitic expression was of particular and dual interest, because (1) the dermal mesenchyme is a derivative of the somites, and (2) the induction of mammogenesis, characterized as a combination of cell elongation and *Wnt10b* expression [60], first manifests itself as a line of fragments overlying the ventral (hypaxial) tips of the somites between forelimb and hindlimb on the flank, which suggests the involvement of hypaxial somitic signals in the onset of mammogenesis [61]. The relevance of the somites in the induction of mammogenesis was supported by the finding that hypaxial truncation of the somites, as in *Pax3* null embryos, is associated with a narrower and dorsally displaced ML on the flank, and delayed formation of MR3 forms compared to wt embryos [60].

In wt embryos, this hypaxial area has the highest *Egf10* expression within the somites. At the time of onset of mammogenesis in wt embryos, *Egf10* is expressed in the somites and limb buds, while the gene encoding its main receptor *Egfr2b* is expressed in the surface ectoderm. *Egfr2b*^{-/-} and *Egf10*^{-/-} embryos do not form a mammary streak/line on the flank, and no MRs (except MR4). By contrast, hypomorphic *Egf10*^{-/m1cy24Lacz} embryos do form a ML and MRs, but not MR3. *Gli3*^{Xt-J/Xt-J} (null) embryos resemble *Egf10*^{-/m1cy24Lacz} embryos with regards to ML and MR3 formation, and have reduced somitic *Egf10* expression levels while *Egf10* expression in the limbs is unchanged or elevated. Stand alone, each of these evidences for somitic involvement in the induction of mammogenesis on the flank is circumstantial. Nonetheless, the combined analysis of mammary phenotypes and gene expression

patterns in these mutants makes a strong case for involvement of somitic signals, i.e., *Gli3* and *Egfr10* in the induction of mammaryogenesis between the limbs [60].

The expression patterns in wt and mutant embryos suggested that FGF10 acts downstream of *Gli3*, but are no proof of such. As FGF10 is a soluble factor, it can be added to culture assays. Implantation of a bead soaked in FGF10 in explant cultures of E11.5 *Gli3^{Xt-J/Xt-J}* embryonic flanks rescued the formation of MR3, indicating that *Egfr10* indeed acts downstream of somitic *Gli3* and is sufficient to induce MR3 in the absence of *Gli3* [60, 189].

Gli3 is a transcription factor with two family members, *Gli1* and *Gli2*. The *Gli1* protein is a transcriptional activator that is usually produced in response to Hedgehog signaling. By contrast, *Gli2* and *Gli3* are often co-expressed at sites with no Hedgehog signaling, which allows their cleavage and consequent functioning as transcriptional repressors. In the presence of high Hedgehog signaling, they can however remain uncleaved and act as transcriptional activators. By replacing two *Gli2* alleles by *Gli1* activator in the absence of one allele of *Gli3*, Hatsell and Cowin were able to restore the *Gli3* mammary phenotype, demonstrating that *Gli3* acts as a repressor [153] as previously predicted [171, 196]. Since the absence of *Gli3* leads to reduced somitic *Egfr10* expression [60], *Gli3* regulates *Egfr10* transcription indirectly.

But how do *Gli3* and *Egfr10* relate to the other somitic/dermal genes, e.g., *Tbx*-genes (Fig. 3)? Around E10.5, wt embryos begin to express *Tbx2* in a band of ventral dermal mesenchyme encompassing the prospective mammary streak between forelimb and hindlimb, and *Tbx3* in a similar but wider band spanning approximately the ventral half of the underlying somites. *Tbx3* is also expressed in the mammary placode epithelium once it is formed. While heterozygous nulls for either gene do not have a mammary phenotype, 20 % of compound *Tbx2/Tbx3* heterozygous nulls have no MR2 at E13.5 (earlier not investigated). This indicates that these *Tbx* genes complement each other or interact with each other via yet unknown mechanisms in early development of MR2 [150]. Wt embryos express *Bmp4* in the ventral dermal mesenchyme in the subaxillary and suprainguinal region at E11-E11.5. The somitic/dermal expression domain of *Tbx3* is narrower in *Gli3Xt/Xt* (null) mutants than in wt embryos [154]. Electroporation of wt flank explants with *Tbx3* downregulates *Bmp4* expression, and broadens the ML. Conversely, electroporation of *Bmp4* downregulates *Tbx3* expression but did not affect the breadth of the ML, while co-electroporation of *Bmp4* and *Tbx3* had the same effect as *Tbx3* alone or caused additional broadening of the ML in the ventral direction. All variables led to an increase of *Lef1* expression as a marker for ME formation. These data indicate a reciprocal negative interaction (direct or indirect) between *Tbx3* and *Bmp4* whose

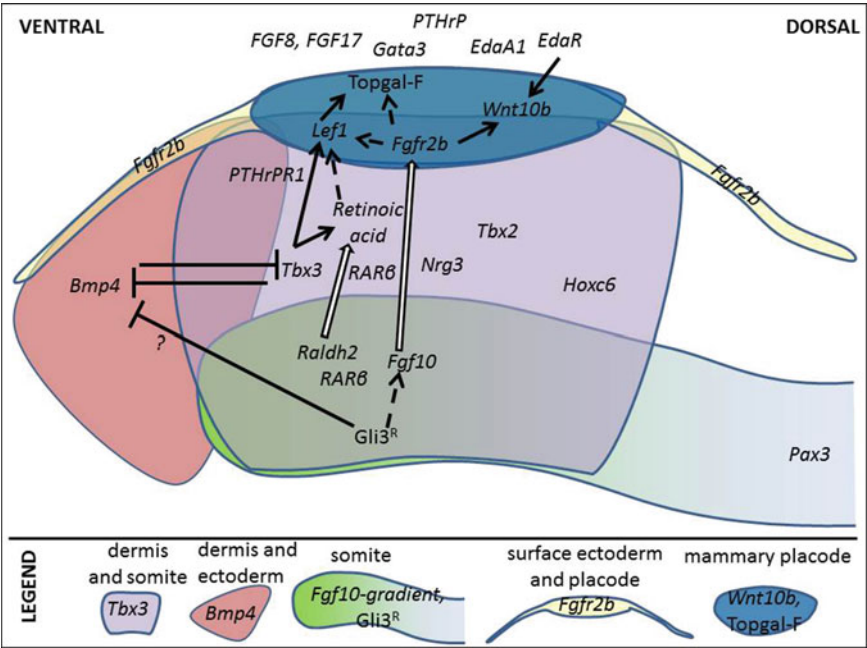


Fig. 3 Molecular players and interactions for the induction of mammary rudiment 3. The molecular cascades regulating the initiation of mammogenesis have been best studied for the mammary streak between the fore limb and hind limb (encompassing MR2, MR3, and MR4) and in particular for MR3, as this MR pair seems most susceptible to loss of gene function and is coincidentally the most accessible for manipulation by for example bead implantation or electroporation in studies with flank explant cultures. This *cartoon* shows the ventral end of somite 15 or 16, with overlying surface ectoderm and developing MR3. *Fgf10* is expressed in a gradient along the somites, with highest expression in the ventral tip, indirectly downstream of *Gli3*-repressor activity. *FGF10* activates the ectodermal *Fgfr2b*, leading to *Wnt10b* expression and Wnt signaling (reported by TOPGAL-F and *Lef1* expression). The site and level of *Fgf10* expression (co-dependent on for example the length of somites which is controlled by *Pax3*) as well as the reciprocal repression between *BMP4* and *TBX3*, likely downstream of *Gli3R*, are determinants of the dorsoventral position of this mammary rudiment. Other molecular players at early stages are indicated in this cartoon as well, although their relationships still have to be determined. Note that other MRs require different tissue and molecular interactions for their induction. Modified from refs. [60, 71, 154, 195], with permission

interface and relative expression levels determine the dorsoventral position and width of the ML [71]. *Gli3^{Xt/Xt}* embryos have a slightly upregulated, dorsalized and posteriorized *Bmp4* expression and correspondingly, the *Tbx3/Bmp4* interface seems to be dorsalized, suggesting that the reciprocal inhibitory interaction between *Tbx3/Bmp4* functions downstream of somitic *Gli3*. Given that *Bmp4* has Gli binding sites, *Gli3* may repress *Bmp4* directly [154]. Interactions of these genes with *Hox* genes, *Nrg3*, and retinoic acid signaling remain to be investigated.

Interestingly, it became clear that at different locations along the ML, the MRs have different requirements for or sensitivities to these mesenchymal factors (Table 1). Despite these differences in mesenchymal inducers, the cellular response in the overlying

ectoderm is the same, being the loss of proliferative activity and gain of migratory activity towards the prospective ML and placode positions [46, 54]. The dorsoventral position of the streak on the flank depends on the length of the somites, and gene activity in the somites, such as *Gli3* and *Egfr10*. At E10, the somites are still small spherical structures adjacent to the neural tube, but within half a day, they start to elongate ventrally and express *Egfr10*. Somitic *Egfr10* levels increase between E10.5 and E11.5, concomitant with the appearance of the mammary streak between the limbs, and the MRs. The ML and all MRs except MR4 are absent in *Egfr10*^{-/-} embryos. FGF10 is known for its chemotactic function in other organs, and as the surface ectoderm expresses its main receptor, FGF10 may exhibit a similar chemotactic function on the surface ectoderm, “dragging” it along to progressively more ventral position until the somites reach the ventral lateral plate mesoderm [60]. This would be consistent with the observations that multilayering of the ML and MRs does not result from cell proliferation, but from cell aggregation or influx [54].

Furthermore, despite the differences in mesenchymal inducers along the mammary line, the known molecular responses of the overlying ectoderm are also the same, namely de novo or increased expression of genes such as *Wnt10b*, *Wnt6* and an engagement in Wnt signaling along the entire ML [61, 148, 191], soon followed by expression of *Lef1* [52], *EdaA1* and *EdaR* [43], *Gata3* [152], *Nrg3* [193] several FGFs [62], and PTHrP [164, 166], specifically in the placode epithelium.

As mentioned, *Wnt10b* first appears as an array of fragments overlying the ventral tips of the somites [61]. However, not all *Wnt10b*^{+ve} fragments use their potential to become a MR. Whether they do, depends in part on the level of somitic *Egfr10* expression, as deduced from the non-induction of MR3 in *Egfr10* hypomorphic mutants. Moreover, *Egfr10* is expressed in a bilateral gradient across somites 12–18, and MR3 is formed above the somites (#15/#16) with the highest *Egfr10* expression in wt embryos [60]. In part, it also depends on sufficient levels of canonical Wnt signaling in the ectoderm, as mutants with a complete reduction in Wnt signaling fail to form the ML and MRs [148], and mutants with a partial reduction of Wnt signaling form MRs with impaired growth and which often regress [135, 147, 155–157, 160–162]. Conversely, increased Wnt signaling by addition of for example Wnt3A to explant cultures, or creating tissue-specific knockouts for inhibitory co-receptors or transgenic overexpression of activators of the pathway, leads to enlarged MRs [148, 155]. By contrast, tissue-specific overexpression of *EdaA1* or its receptor, or *Nrg3* [159] leads to conversion of more *Wnt10b*^{+ve} fragments into MRs [85, 144]. *Nrg3* seems to regulate migration of mammary epithelial precursors towards the placode sites [180], whereas *Eda*/TNF signaling

represses ectodermal Wnt signaling and enhances mesenchymal Wnt signaling at E13.5 [57]. It is now of interest whether the same interaction exists between Eda and Wnt signaling pathways at the induction stage. In any case, by regulating the size and number of MRs, they are important determinants of the patterning of ME in the ectoderm.

One role for Wnt-signaling may be to downregulate the proliferative activity of ectodermal cells in association with their acquisition of a ME fate. This conclusion is based on data from Ahn et al. [155] who show that MR2 and MR3 fuse in the absence of the Wnt-antagonists Lrp4 or Wise, preceded by a loss of proliferation in the interplacodal region.

Notably, the various ligands, receptors and antagonists of Wnt signaling vary widely in their expression domain, from broad expression in the dermal mesenchyme or surface ectoderm, to restricted expression in the ventral or dorsal domain or flank, ML, rudimentary ME or MM. Thus, various modes or subsets of canonical Wnt signaling may exist in the mammary region, both in the epithelium and in the mesenchyme. This is exemplified by the different expression patterns in MRs [60, 148, 153–157, 160–162, 166] as well as in other organs [197] of the reporters for canonical Wnt-signaling: Topgal-F [179], Topgal-C [181], Batgal [182], and Axin2-LacZ [198] (Table 2). It remains a challenge to identify separate roles for mesenchymal and epithelial Wnt-signaling, let alone whether subsets of Wnt signaling locally act alone or in concert with other subsets.

8 Molecular Regulators of Growth and Survival of the MRs Until E16

While a complete abolishment of Wnt-signaling through overexpression of the inhibitor Dkk1 prevents the formation of all MRs [147, 148], MRs are induced if Wnt-signaling is only partially reduced due to a null mutation for *Lefl*, *Lrp5*, or *Pygo2* [135, 156, 157, 161]. However, such MRs are small, grow poorly if at all, and may regress with variable penetrance before E15.5. Whereas *Msx1*^{-/-} single knockouts have no embryonic mammary defects and MRs in *Msx2*^{-/-} develop normally until sprouting stage, *Msx1*^{-/-}; *Msx2*^{-/-} double knockouts develop MRs that fail to express *Lefl* and regress by E15.5 [168].

As *Lefl* is a transcriptional target and mediator of Wnt signaling, it is tempting to speculate that the regression in *Msx1*^{-/-}; *Msx2*^{-/-} mutants is due to reduced Wnt signaling; perhaps because ectodermal cells retain their proliferative activity and fail to acquire a mammary fate or commit to it if Wnt signaling is low. However, in wild types, *Lefl* expands its expression domain from the ME to include the MM by E15.5 [67] while it mediates the converse expansion of Topgal-C expression (a reporter for a subset of Wnt

signaling) from the MM to include the ME between E13.5 and E15.5 [156]. Notably, during this time span, the epithelial compartment of the wt MR transits from growth by epidermal cell recruitment to growth by proliferation of the ME cells proper [46, 54]. It is thus possible that the absence of Wnt-signaling in the ME in E15.5 *Lef1*^{-/-} embryos also disrupts the functional transition that ME cells need to undergo around that time, leading to lack of growth and eventual regression of the MRs.

Interestingly, loss of p190B-RhoGAP allows for MR induction, but at E14.5 the buds are small despite a slight increase in epithelial proliferation and a lack of apoptosis [163]. Given that p190RhoGAP is expressed in the ME of E12.5 embryos onwards, and that the enzyme is known for its roles in cytoskeletal remodeling to promote cell migration and inhibit mitosis, it is conceivable that the mammary phenotype in *p190B-RhoGAP*^{-/-} embryos is caused by both impaired cell migration and sustained cell proliferation.

Contrary to *Tbx3*^{-/-} embryos, *Tbx3*^{+/-} embryos induce all five MR pairs, but the three thoracic pairs are often lost between E13.5 and E18.5. This defect is exacerbated in *Tbx2*^{+/-};*Tbx3*^{+/-} double heterozygotes [150]. Both genes are well known for their role in cell cycling control through p19^{Arf}/p53 signaling. While this mechanism is intact in these mutants, it remains of interest to investigate which signaling cascade is impaired and causes the haplo-insufficiency in mammary development of these mutants [150].

9 Molecular Regulators of Sexual Dimorphism

As mentioned far above in section 5 about steroid hormones, the sexual dimorphism of mammary gland development is created by the absence or presence of androgen receptor activation in the mammary mesenchyme of female respectively male mice [49, 50, 63, 106, 107, 114]. Analysis of null mutants for the genes encoding PTHrP or its receptor PTHrP-R1 revealed their lack of sexual dimorphism in mammary gland development: Mammary glands in these mutant males and females lack androgen receptor and tenascin-C expression in the MM, and develop similar to their counterparts in wild type female embryos [84, 109]. In wild type mice, *PTHrP* is expressed in the ME from placode stage onwards, while *PTHrP-R1* becomes broadly expressed in the dermal mesenchyme [84, 109]. These expression patterns may suggest that the defect in mutants is due to an absence of PTHrP/PTHrP-R1 signaling between the ME and prospective MM. However, far prior to the onset of mammary gland development, PTHrP and PTHrP-R1 are expressed in several extra-embryonic and embryonic tissues [199]. Therefore, further testing was required to exclude the possibility that the mammary defect is a secondary effect of lack of PTHrP/PTHrP-R1 signaling earlier in embryogenesis.

Rescue experiments in which PTHrP was reintroduced in the ectoderm/epidermis of *PTHrP*^{-/-} embryos by crossing in a Krt14-PTHrP transgene, restored androgen receptor expression. This facilitated the androgen response in male mutants. These experiments confirmed that the mammary defect is indeed caused by the absence of PTHrP signaling in the MR proper, and that no earlier PTHrP signaling is required [109]. Similarly, *PTHrP*^{-/-} and *PTHrP-R1*^{-/-} mutant mice lack Topgal-F expression (a marker for a subset of Wnt signaling) in the MM. Conversely, transgenic over-expression of PTHrP in the entire flank induces ectopic Topgal-F expression in the underlying dermal mesenchyme, confirming that mesenchymal Wnt signaling requires no PTHrP/PTHrP-R1 signaling prior to mammary placode formation.

Epistasis assays in which the Wnt-transducer β -catenin was removed from the mesenchyme in Krt14-PTHrP transgenic mice, showed that dermal β -catenin is required downstream of PTHrP/PTHrP-R1 signaling between the mammary placode epithelium and its contiguous dermal mesenchyme, to induce mammary mesenchymal specific markers such as Wnt signaling and expression of *lef1*, estrogen receptor and androgen receptor [166]. Like PTHrP/PTHrP-R, also *Gli3* is required for androgen receptor and tenascin-C expression, and it now becomes interesting to study if and how PTHrP/PTHrP-R signaling, Wnt signaling and *Gli3* interact to regulate expression of androgen receptor and tenascin-C as differentiation markers for MM [154]. Despite a normal testicular histology and androgen receptor expression in the MM, the MRs of some Krt14-*Eda* males may escape the androgen-mediated destruction and even form a nipple. The ME manages to sprout and enter the fat pad precursor, where it undergoes a modest degree of branching morphogenesis, albeit with a lack of canalization. Most likely the escape from destruction is provided by precocious proliferation of the ME, which allows penetration into the androgen receptor negative fat pad precursor [57]. Remarkably, there also exists a sexual dimorphism in sensory innervation of the mammary gland. This is due to the expression of a truncated form of *TrkB*, a receptor for the neurotrophic factor BDNF, downstream of androgen receptor activation. This truncated receptor prevents normal BDNF/*TrkB* signaling in sensory axons, which leads to a loss of innervation of the mammary gland in males [167].

10 Molecular Regulators of Nipple Formation

The nipple is a late appendage to the skin and mammary gland, both in terms of evolution and in embryonic development, as they only develop in marsupials and placentals, and as a secondary

structure to the mammary gland [6]. The supernumerary MRs in *Krt14-EdaA1* transgenic mutants do form nipples, albeit it with an aberrant shape, and not all connected to a ductal network and associated with a fat pad [85, 144]. Nipples of *Eda^{Ta/Ta}* (null) mice were abnormally flat, but nonetheless both the loss and gain of function mutants nursed their offspring normally [144]. *PTHrP^{-/-}* and *PTHrP-R1^{-/-}* mutants do not develop nipples, nor can their nipple development be rescued with transgenic Krt14-PTHrP [67, 169, 200]. However, the entire ventral epidermis transforms into nipple skin when transgenic Krt14-PTHrP is expressed on a wt background, ectopically in the entire ventral epidermis instead of in the ME only [67]. These analyses led to the conclusion that PTHrP/PTHrP-R1 signaling is required and instructive for nipple development. Normally, PTHrP-R1 is ubiquitously expressed in the ventral dermal mesenchyme, whereas PTHrP expression is restricted to the ME only. Thus, despite the ubiquitous expression of PTHrP-R1 in wt embryos, activation of this receptor is restricted to just a few layers of mesenchyme in close proximity to the ME. This mesenchyme differentiates into MM and signals back to the overlying epidermis, which responds locally by differentiating into nipple skin [67]. As PTHrP/PTHrP-R1 signaling activates Wnt signaling to specify the MM [166] it is perhaps not surprising that mutants lacking the Wnt co-receptor Lrp6 have smaller nipples [162].

One feature of nipple formation is the suppression of hair follicle formation. Indeed Krt14-PTHrP transgenic embryos lack hair follicles on their ventral (nipple) skin [201], in conjunction with reduced BMP signaling due to reduced transcription of the BMP receptor BMPRI1A [164]. Loss of *Msx2* in *Krt14-PTHrP* embryos (*Krt14-PTHrP;Msx2^{-/-}* mutants) rescues hair follicle formation. As BMP4 and PTHrP have a synergistic stimulatory effect on *Msx2* expression in cultured dermal mesenchymal cells, it was concluded that *Msx2* mediates the repressive effect of PTHrP/PTHrP-R1-augmented BMP signaling on hair follicle development in the nipple area [164]. Indeed, suppression of BMP signaling by transgenic expression of *Krt14-Noggin* allows the formation of *Shh*-expressing hair follicles in the nipple area [202]. Moreover, transgenic Noggin suppresses *PTHrP* expression, whereas addition of BMP4 to cultured cells augments PTHrP-promoter activity [202]. This points to a feed-forward loop between PTHrP and BMP signaling. In the absence of the Gli3 repressor of (sonic) Hedgehog signaling, hair follicles develop in the nipple area [154]. It is now of interest to study the relationship between PTHrP, BMP, and Hh signaling in establishing a properly differentiated nipple tissue without hairs.

Interestingly, the time frame allowing nipple development is very wide, as supernumerary nipples are formed in *Sostdc1*^{-/-} mice at the end of puberty around 6 weeks postpartum [160]. Remarkably, these nipples are not connected to a ductal network, and both the normal and supernumerary nipples contain hair follicles [160].

11 Molecular Regulators of Sprouting and Branching Morphogenesis

In reduction or loss of function mutants for *PTHrP*, *PTHrP-R1*, *Msx2*, *Egfr10*, *Tbx2/Tbx3*, *Pygo2*, *Lrp6*, *Gli3*, or *Eda*, and in transgenic mice overexpressing the super-repressor of *Eda*/NFkB signaling, *IkbαΔN*, mammary buds all fail to properly elongate into sprouts or are impaired in branching morphogenesis [44, 52, 54, 57, 150, 157, 162, 168]. In wild types, all these molecules are expressed in the MM and/or fat pad precursor, with exception of *Pygo2*, *Lrp6*, the *Tbx* transcription factors, and *PTHrP* which is expressed in the ME but finds its receptor in the MM. It was therefore likely that sprouting and branching morphogenesis of the ME are regulated by molecular interactions of the ME with its surrounding mesenchymal tissues. This has been tested and validated for *PTHrP* and *FGF10* signaling: *Egfr10*^{-/-} ME was able to generate a branched tree when grafted into a cleared fat pad of a 3-week-old wt [52]. Similarly, tissue recombinants of E13.5 *PTHrP-R1*^{-/-} ME with wt MM that were grafted under the kidney capsule, did show ductal outgrowths similar to wt/wt recombinations, while recombinants of wt ME with *PTHrP-R1*^{-/-} MM did not grow out [84]. These data showed that *FGF10* and *PTHrP-R1* expression are only required in the mesenchyme for normal branching. The level and timing of *PTHrP-R1* activation is important, as transient overexpression of *PTHrP* in the epidermis using the *Krt14*-driven inducible tet-off system [203] during prenatal branching morphogenesis causes branching defects during puberty [170]. *PTHrP*/*PTHrP-R1* signaling regulates *Msx2* expression in the MM [164], and the similarity in sprouting and branching defects in null mutants for *PTHrP*, *PTHrP-R1*, and *Msx2* suggests that *Msx2* is a mediator of *PTHrP*-induced sprouting and branching. Overexpression of *Eda* in *Krt14-Eda* transgenics induces precocious branching. Microarray expression profiling of *Eda*^{-/-} skin cultured in the absence or presence of recombinant *Eda*, showed an upregulation of amongst others *Wnt10b* and *PTHrP* in response to *Eda*. In accordance, higher levels of these mRNAs were detected by in situ hybridization of *Krt14-Eda* embryos. In an ex vivo explant culture setup adapted to monitor branching morphogenesis [89], recombinant *Wnt3a* and *PTHrP* accelerate branching morphogenesis in mammary. It is therefore likely that *Eda*

promotes branching morphogenesis via its regulation of PTHrP and Wnt signaling [57].

Other evidence for an involvement of Wnt signaling in branching morphogenesis comes from the severely impaired ductal branching in constitutive and skin-specific null mutants for *Pygo2* [157] and *Lrp6*^{-/-} mutants [162].

Tbx2 and *Tbx3* are expressed in the mesenchyme surrounding the nipple sheath, and *Tbx3* but not *Tbx2* is also expressed in the mammary epithelium at E18.5. Heterozygous *Tbx2* nulls have no mammary defects, but heterozygous *Tbx3* nulls display reduced branching in all their MRs at E18.5. Whereas double heterozygotes for both genes more often lose MR1–3 between E13.5 and E18.5, the branching defect in the rudiments that do survive is not more severe than in *Tbx3* heterozygotes [150].

12 Embryonic Mammary Gland and “Omics”

With a modification of Kratochwil’s enzymatic tissue separation technique [82, 88, 204], the ME and MM of MR4 of several E12 embryos have been isolated and pooled per tissue for the subsequent extraction of mRNA and transcriptome analysis [29]. RNA was then amplified and subject to microarray analysis. By comparing the transcriptional profiles of both tissues with that of a non-treated intact MR (ME + MM), the gene pool that was activated by the enzyme treatment could be filtered out, and relevant transcriptome profiles were obtained with many new potential regulators of early mammogenesis. Interestingly, the ME profile showed many similarities with the mammary stem and progenitor cell populations of adult mammary gland #4 [29], and subsets of its profile also showed similarities to breast cancer profiles [205]. With similar tissue isolation techniques, the expression of miRNAs was also analyzed and led to the discovery of miR206 in the mammary mesenchyme [206]. Overexpression of miR206 by electroporation in flank explant cultures led to significant changes in gene expression in the MM, amongst others a reduction of estrogen receptor expression [206].

To reduce the effect of enzyme treatment on gene expression profiles and to speed up the tissue separation and processing time for increased RNA integrity, Sun et al. developed a tissue separation and harvesting technique based on the dehydrating effect of RNA-Later [86]. Analysis of these tissues have revealed that each of the five MRs has different expression profiles [ref. Sun and Veltmaat unpublished, <http://www.veltmaatlab.net/research.html#sunli>]. Any regulatory role of these differentially expressed genes in the identity of the MRs needs yet to be established.

13 Stem Cell Activity in the Embryonic MR

In 1979, Sakakura transplanted an E13.5 MR into the fat pad of a prepubertal mouse and demonstrated that the transplant could grow out, branch, and produce milk like an endogenous mammary gland [92]. With the exception of testing for milk production, similar outgrowth potential has been observed for intact E12.5 MRs [18]. These outcomes indicate that cells of the E12.5 ME have a pluripotent capacity and enormous proliferative potential, possibly via self-renewing stem or progenitor cells.

The intron5/6 region of the gene encoding Ship1 phosphatase contains stem-cell specific promoter activity [186]. Interestingly, this transgenic promoter construct drives GFP expression even in the ML and uniformly in the MRs at E11.5 and E12.5 ([180, 186] and cover illustration of [41]), suggesting the presence of mammary stem cells from the onset of mammogenesis onwards. In that context, it is of interest that (1) Wnt signaling is required for the induction and development of the embryonic mammary gland [191], as well as for self-renewal of mammary stem cells in the adult [207]; and that (2) Pygo2, which converges with Wnt-signaling, is enriched in adult mammary stem cells and required for proper induction and development of the embryonic MRs [157].

The phenotypic identification of mammary stem cell populations began in adult mammary glands, by fluorescence-activated cell sorting (FACS) of single cell suspensions of partial mammary glands. This technique is based on fluorescent labeling of tissue-specific cell-surface markers, which facilitates the separation of mammary epithelial cells from endothelial and stromal cells. Epithelial subpopulations can be further sorted based on fluorescent labeling of subpopulation-specific markers, and transplanted in limiting dilutions into cleared fat pads of prepubertal mice, to be scored for mammary repopulation units (MRUs) in these fat pads. Such studies identified a high MRU-potential of the CD24^{high};CD49^{high} subpopulation, whose regenerative potential was demonstrated by their ability to generate daughter MRUs upon retransplantation to a new cleared fat pad [208, 209]. This technique has recently been used to identify subpopulations with high MRU-capacity in the E18.5 ME [17–19]. These studies demonstrated that the stem cell activity of the embryonic ME resides entirely in the CD24^{high};CD49^{high} subpopulation, and that embryonic ME has a higher regenerative potential than adult ME [17–19].

However, when single ME cells of an embryonic MR are transplanted in a cleared fat pad, they rarely generate mammary glands. Moreover, when the donor embryo is younger than E15.5, outgrowths are only observed when the ME cells are co-transplanted with Matrigel. Perhaps this can be explained by lineage-restricted

stemcellness at E12.5 as follows: Cells can be labeled in a tissue-specific manner and under temporal control by combining the *CreERT2* and *mT/mG* transgenes. The *mT/mG* transgene (encoding the fluorochromes Tomato-Red and Green Fluorescent Protein, GFP), can be inserted in for example the *Rosa26* (*R26R*) locus for ubiquitous expression. Under normal conditions, such transgenic cells express Tomato-Red, whereas upon exposure to Cre-recombinase (from the *CreERT2* transgene), they switch to GFP expression. The *Cre-ERT2* transgene expresses Cre-recombinase upon occupation of its ERT2 binding sites by estrogenic compounds like tamoxifen. Insertion of this transgene in the locus of a tissue-specific gene and temporal control of administering tamoxifen provides temporospatial control of the color switch of *mTmG* transgenic cells, and subsequently all progeny of switched cells will express GFP. *Axin2* is a mediator and target of Wnt/ β -catenin signaling, and is expressed throughout the MR epithelium at E12.5 [183]. When female mice pregnant of *Axin2*^{CreERT2/+}; *R26R*^{mTmG/+} embryos are given tamoxifen mice on the 12th, 14th or 17th day of pregnancy, the mammary glands of their offspring in adulthood will only express GFP in luminal cell, indicating that embryonic mammary cells engaged in canonical Wnt signaling are progenitors for exclusively the luminal lineage [183]. These data suggest there may already be separate stem or progenitor cell populations for the luminal, the basal, and perhaps both cell layers at that time. This lineage restriction of at least some cells in the embryonic MR may explain the low take rate of transplanted single cells of embryonic MRs. On the other hand, transplantation of FACS-sorted lineage-restricted stem cells in cleared fat pads still yields normal outgrowths with a basal and luminal compartment, strongly suggesting that lineage-restriction is a facultative state in real life, which can be converted into bipotency upon disturbance of the normal cell and tissue integrity [183].

The success rate of generating a mammary gland increases dramatically when single ME cells are used of E15.5 and E16.5 MRs, and keeps on increasing by using E17.5 and E18.5 ME. These data suggest that critical properties required for the outgrowth of a mammary gland in such experiments are required at E15.5 [18]. It is worth noting that E15.5 is also the decisive stage for MRs in certain mutants (e.g., *Lef1*^{-/-}; *Msx1*^{-/-}; *Msx2*^{-/-}) to either survive or revert to an epidermal fate [135, 156, 168], just prior to keratinization and impermeabilization of the epidermis. As ME cells are thus not committed to a mammary fate prior to E15.5, an alternative explanation for the low take rate of single ME cells in transplantation assays, it that the harsh circumstances during cell dissociation may change their expression pattern such that they cannot maintain their identity as mammary stem cells of any kind [41].

Microarray analysis of this subpopulation revealed that the gene expression profile of E18.5 fetal mammary stem cells (fMaSCs) cells shows overlap with, but is very different from that of adult MaSCs. By contrast, the expression profile of fetal mammary stroma (fSTR) more closely resembles that of aMaSCs. fMaSCs express markers of multiple adult mammary lineages (indicating multipotency) in addition to gene sets that are unique for embryonic ME [18]. Although the expression signatures of fMaSCs and fSTR are significantly different from those of E12.5 ME respectively MM, it is of great interest that some breast cancer subtypes are enriched for any of these profiles [18, 29]. However, it must be noted that the entire experimental procedure prior to the gene-profiling step may have altered the expression pattern, given the observed differences in potential (bipotent versus lineage-restricted) observed for the same cell population in lineage-tracing experiments versus FACS + transplantation assays [183].

14 Experimental Design and Pitfalls in Interpretation of Own and Published Data

Studies on the embryonic mammary gland rely partly on distinct techniques, some of which differ from those in the adult mammary gland, such as explant culture, tissue separation and recombination, grafting, whole mount in situ hybridization, and immunodetection. The development and applications of those techniques has been described in this review. Figure 4 illustrates how these field-specific techniques can be combined in parallel or sequentially with generic molecular and biochemistry techniques, as well as with the most recent stem cell and “omics” techniques, to address most questions related to embryonic mammary gland development.

Until about the 1970s, only few experimental interventions were possible, and consequently most studies were based on histology and microscopy solely. Such studies revealed differences in histological appearance and organ morphology between different species and developmental stages. However, some researchers would speculate or draw conclusions about possible mechanisms that would cause these appearances and changes, without having the proper experimental basis for such conclusions. Some of these conclusions were wrongfully propagated in the literature and extrapolated to other species, and almost became dogmatic to the field. For example mammary gland development was published to start with the formation of a continuous ectodermal band/line/ridge from and on which the MRs develop [45]. However, recent studies with molecular techniques and genetically engineered mice with more than the usual five pairs of MRs, contradict this: First many individual sites of possible MR development are formed, which then temporarily fuse into a continuous line (one line on each flank),

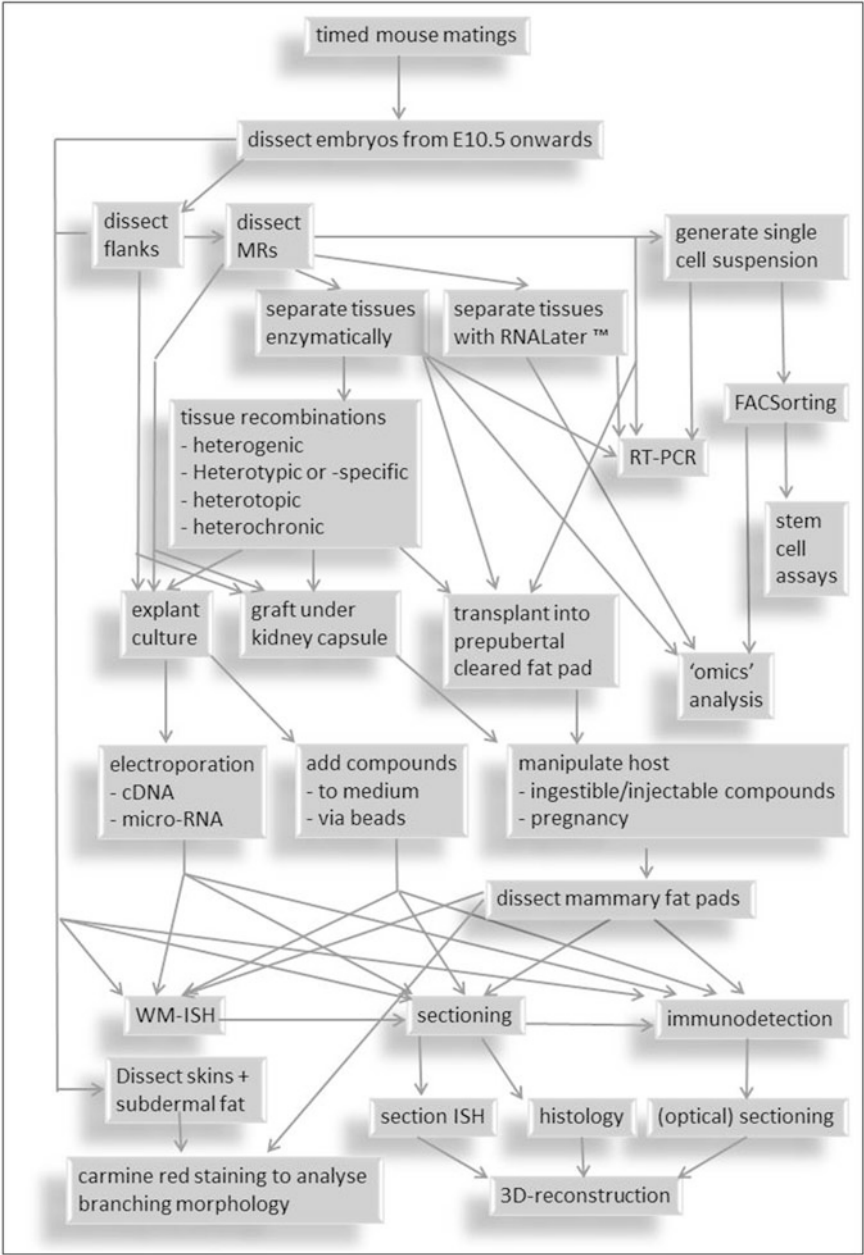


Fig. 4 Flowchart for experimental setup for studies of embryonic mammary development. These studies start with the husbandry of (genetically engineered) mice, and may include explant culture, and a variety of molecular and histological analyses

after which MR development continues only at a subset of the initial sites [41, 61]. The histological observation that the ML and MRs were already multilayered before the surface ectoderm, led to a similar unfounded conclusion that this precocious multilayering was due to locally enhanced cell proliferation [45]. Decades later, a study with tritiated thymidine incorporation demonstrated the near absence of proliferative activity in MRs between E12.5 and E13.5 [13]. In subsequent literature, these two conclusions were combined and propagated as the misconception that MRs would undergo 24 h of proliferative arrest - after supposedly initial high proliferative activity - between E12.5 and E13.5, even though Balinsky had already contested the assumption that initial multilayering was due to cell proliferation [46]. These examples underscore two often-made mistakes: drawing mechanistic conclusions from static data without experimental variables, and the wrongful combination and rephrasing of published conclusions.

Nowadays, gene and protein expression data are often similarly misinterpreted. For example, whole mount in situ hybridization patterns are often judged without sectioning. However, due to the transparency of the embryo, hybridization signals of deeper tissues can be seen through the embryonic skin, but not attributed to a particular organ or tissue. It may be tempting to interpret a stacked array of dorsoventral hybridization stripes on the flank as somitic gene expression, while it is also possible that the signal is generated by the somite-derived dermis or overlying ectoderm. Only sectioning of the embryo can reveal which (combination) of these tissues generates the hybridization signal.

In addition, the absence of a hybridization signal is often interpreted as the absence of a structure, e.g., the absence of *Wnt10b* or *Lef1* expression as markers for MRs, is often interpreted as an absence of MRs. This implies that the researcher assumes that these markers are required for the formation of MRs. This assumption is understandable, since Wnt signaling is known to be required for placode formation [148]. Nonetheless, the assumption is incorrect. Whereas *Wnt10b* is a very suitable marker for the ML and MRs of C57BL/6J mice [61], some albino mouse strains do not express this marker yet develop functional mammary glands (J. Veltmaat, unpublished observations) and *Wnt10b* null mice have no reported mammary gland defect [210]. This illustrates that mere gene expression should not be confused with (or misinterpreted as) gene function, and an absence of gene expression may not be interpreted as an absence of a structure. Similarly, *Lef1* is a marker for and mediator of canonical Wnt-signaling. Whereas MRs of *Lef1* nulls show severe hypoplasia at E12.5 [156] and arrest in bud stage or disappear by E15.5 [135], all MRs are induced at E11.5 [pers. comm Kratochwil in ref. [53]; and personal observations]. Therefore, an absence of *Lef1* expression should not be

interpreted as an absence of MR formation. TOPGAL-F is a suitable reporter for only a subset of all Wnt-signaling. Even though it also nicely marks the ML and MRs, its absence of expression does not necessarily indicate an absence of all Wnt signaling or MR formation. The absence of marker expression should always be accompanied by histological analysis to warrant a conclusion that structures are indeed absent.

Conversely, the presence of gene-expression may indicate that a structure is there, but does not necessarily mean the structure is normal. In some mutant mouse strains on a TOPGAL background, the MRs may appear as narrower or wider dots, which is often interpreted as smaller or larger MRs. However, the size but not morphology may still be normal, as the MRs may have a relatively elongated respectively flattened shape compared to wild type littermates. In conclusion, it is always advisable to combine gene expression analysis with histological analysis.

If new mutant mice are generated and published “with no mammary defects” or “to nurse their offspring normally,” this does not exclude possible anomalies in the number, morphology, or full functionality of mammary glands, especially if the publishing lab has no interest in mammary development *per se*.

Only since the beginning of this century has the notion grown that all pairs of MRs in mouse embryos are different with respect to the timing of their appearance [52, 53, 62], their molecular requirements and morphogenetic program [27, 52–54]. When reading older literature, but even when reading recent literature, one should keep in mind that findings and models may be published as if valid for all MRs, while perhaps only one, two, or three pairs of MRs were used for the study without specific mentioning. MR3 is especially easily accessible for experimentation; whereas MR1 and MR5 are hidden behind the limbs and hard to view or retrieve, and consequently are often not taken along in the analysis. Thus, if a publication states that for example embryonic mammary glands of embryonic lethal mutants develop with—or without—abnormalities upon transplantation in a cleared fat pad, this may not hold true for all MRs. On the same note, it is advisable to design future studies such, that all MRs are examined separately in each experiment, and reported as separate entities in the literature as well.

Modern techniques are becoming increasingly sensitive, allowing even stem cell assays and transcriptome analysis to be performed with embryonic mammary rudiments.

A few technical territories remain unexplored, such as proteomics and biochemical assays such as immunoprecipitations or pull-down, due to their requirement for greater quantities of sample material. But a true technical challenge seems to be live imaging of cell behavior during embryonic mammary gland development, due to the continual shift of the plane of

interest during growth *ex vivo*. The establishment of good live imaging protocols would be extremely helpful in establishing the area and direction of cell migration in the establishment of the ML and MRs, or the behavior of cells within the developing MRs.

15 Conclusion

This review describes how, with perhaps the exception of some live imaging and biochemical techniques that require large amounts of protein as input, all techniques that are used to study the postnatal mammary gland can also be used to study the embryonic mammary gland. But the embryonic mammary gland has other advantages: It can be easily dissected, and optionally its tissues can be separated and recombined in various combinations, for growth *ex vivo* or as a transplant, which facilitates the study the role of tissue interactions in morphogenesis and function. Such studies are more difficult to carry out with adult mammary glands, due to their greater tissue complexity. Moreover, in cases where the role of a gene or its mutation in the postnatal gland cannot be studied due to perinatal lethality of constitutive mutants, and tissue-specific mutants are not available, mutant embryonic MRs can be transplanted into a wild type prepubertal mammary gland for further study.

Studies on the embryonic mammary gland are certainly relevant to postnatal mammary gland development, function, and pathology, because the embryonic mammary gland displays many features of the postnatal mammary gland: It already contains stem cells [17–19], commits to a mammary fate by producing milk when stimulated by pregnancy hormones [92], and undergoes a series of morphogenetic changes that are reiterated during puberty and pregnancy. There is a high degree of similarity in tissue interactions and molecular controls of these changes during embryonic and postnatal life in the mouse [13, 16]. Moreover, such molecular similarities have also begun to be discovered between mice and human, even extending between murine embryonic mammary development and postnatal mammary tumorigenesis in mouse and human [211]. Another resemblance lies in the influence of the mammary stroma on the functional differentiation and homeostasis of the mammary epithelium during embryonic mammary gland development, and postnatal formation of hyperplasia and neoplastic lesions [30, 90, 212]. Such parallels make studies of the embryonic mammary gland important even beyond the questions concerning the embryonic phase *per se* [38].

In conclusion, given the relative lack of tissue complexity of the embryonic mammary glands and the ease with which they can be accessed and manipulated for study, the embryonic mammary

glands are a very suitable starting point or alternative or additional model to study a wide range of questions pertaining to normal and pathological postnatal breast development as well.

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