



**Università
di Brescia**

DIPARTIMENTO DI MEDICINA MOLECOLARE E TRASLAZIONALE

**DOTTORATO DI RICERCA IN GENETICA MOLECOLARE,
BIOTECNOLOGIE E MEDICINA SPERIMENTALE**

Settore Scientifico Disciplinare:
Area 06 - Scienze Mediche
MED/03 Genetica Medica
MED/46 Scienze Tecniche di Medicina di Laboratorio

XXXVIII CICLO

**Development and Optimization of Human iPSC-Derived Mammary
Gland Organoids as an *In Vitro* Platform for Breast Cancer Research**

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ACKNOWLEDGEMENTS

All praises and deepest thanks be to **God Almighty**, for His boundless grace throughout every chapter of my life, and for bestowing upon me the strength, patience, and capability to bring this work to its completion.

I wish to express my most sincere and heartfelt gratitude to my esteemed PhD supervisor, **Prof. Silvia Clara Giliani**, for her exceptional guidance, unwavering dedication, and the remarkable generosity with which she shared her wisdom and expertise throughout this study. I am deeply indebted to her for her invaluable suggestions, her meticulous evaluation of this thesis, and for the countless opportunities for personal and professional growth she so graciously provided. Her mentorship has left an indelible mark upon my academic life.

I extend my warmest thanks to my esteemed colleagues, **Dr. Rosalba Monica Ferraro**, **Dr. Cinzia Caprio**, and **Dr. Elena Mazzoldi**, as well as all the dedicated staff of the Angelo Nocivelli Institute for Molecular Medicine, Civil Hospital of Brescia, for their generous support, encouragement, and the collegial warmth that made this research a truly enriching experience.

I am most grateful to **Prof. Alessandro Barbon**, the current Ph.D. Coordinator, **Prof. Eugenio Monti**, the former Ph.D. Coordinator in the Department of Molecular and Translational Medicine at the University of Brescia, and **Dr. Fabiana Farro**, Ph.D. Officer in Charge, for their continued institutional guidance and encouragement throughout this programme.

Finally, and most deeply, I turn to those dearest to my heart. To the beloved **soul of my Mother** — no longer with me in this world, yet ever-present in spirit — I dedicate my most profound and eternal gratitude. May God shower her soul with His endless mercy and grace. She believed in me before I believed in myself. To my dear **Father**, whose unconditional love and unwavering faith in my abilities have been the most enduring foundation of my strength, I am eternally grateful. And to my cherished **Wife**, whose love, patience, and quiet sacrifice have been my truest source of resilience throughout this journey — I owe more than words could ever express.

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ABSTRACT (*Italian*)

La ghiandola mammaria umana è un organo complesso e responsivo agli stimoli ormonali, il cui epitelio è organizzato in due popolazioni cellulari principali: le cellule luminali alveolari, deputate alla secrezione del latte, e le cellule baso-mioepiteliali, che forniscono supporto strutturale e favoriscono la secrezione latte. Questi compartimenti sono immersi in un microambiente stromale che svolge un ruolo cruciale nella regolazione dello sviluppo e dell'omeostasi ghiandolare. Il carcinoma mammario, la neoplasia più frequentemente diagnosticata nella popolazione femminile mondiale, insorge spesso in seguito ad alterazioni di questa organizzazione cellulare finemente regolata; in questo contesto, le mutazioni germinali nei geni *BRCA1* e *BRCA2* conferiscono un rischio oncogeno significativamente elevato. Pertanto, lo sviluppo di modelli umani *in vitro* fisiologicamente rilevanti e capaci di ricapitolare l'architettura del tessuto mammario riveste una fondamentale importanza sia scientifica che clinica.

Per superare i limiti intrinseci dei sistemi di coltura bidimensionali convenzionali, questo studio presenta un protocollo multi-step ottimizzato per la generazione di organoidi tridimensionali simil-mammari a partire da cellule staminali pluripotenti indotte (iPSC) umane, derivate sia da donatrici sane sia da individui portatori di mutazioni *BRCA1/2*. Inizialmente, le iPSC sono state aggregate in corpi embrioidi mammari (mEB) utilizzando il terreno MammoCult integrato con idrocortisone ed eparina, al fine di promuovere la specificazione verso l'ectoderma non neurale. Successivamente, l'impegno verso il lignaggio mammario è stato indotto mediante la proteina correlata all'ormone paratiroideo (pThrP); i mEB al decimo giorno sono stati poi inclusi in una matrice composita di Matrigel e Collagene I, arricchita con FGF10 e HGF per guidare la morfogenesi ramificata e il differenziamento alveolare. Organoidi strutturalmente organizzati sono stati ottenuti in modo riproducibile entro il trentesimo giorno di differenziamento.

La caratterizzazione molecolare e istologica ha confermato l'avvenuta acquisizione del destino cellulare mammario. Le analisi di RT-PCR quantitativa e immunoistochimica hanno mostrato una progressiva riduzione dell'espressione dei marcatori di pluripotenza (OCT4, NANOG, KLF4), accompagnata dall'aumento dell'espressione di marcatori luminali (GATA3, CK18) e basali/mioepiteliali (TP63, CK5/6, CK14, SMA), riproducendo la caratteristica architettura a doppio strato della ghiandola nativa. È stata inoltre rilevata un'espressione residua dei marcatori

dell'ectoderma neurale PAX6 e FOXG1, indice di una persistente eterogeneità cellulare che rappresenta tuttora una sfida per l'ottimizzazione futura del protocollo.

Un risultato fondamentale di questo lavoro riguarda la regolazione dinamica dei recettori degli ormoni steroidei durante lo sviluppo degli organoidi. In linea con la recente dimostrazione che il recettore del progesterone (PR) viene espresso costitutivamente nelle iPSC in seguito alla riprogrammazione delle cellule somatiche — indipendentemente dalla cellula di origine o dal metodo di riprogrammazione, e in assenza del recettore alfa degli estrogeni (ER α) — questo studio ha monitorato longitudinalmente l'espressione di PR nelle diverse fasi di differenziamento. Il PR è stato transitoriamente down-regolato durante la fase iniziale dei mEBs e successivamente ripristinato negli organoidi maturi a 20 giorni, rispecchiando la sua regolazione fisiologica durante la morfogenesi mammaria. La concomitante espressione di PR e GATA3 negli organoidi maturi riflette un asse regolatorio funzionale, cruciale per il differenziamento luminale e frequentemente alterato nel tumore al seno. Il recettore ER α non è stato rilevato, in accordo con la sua nota riduzione dell'espressione nell'epitelio mammario in attiva proliferazione. Queste dinamiche recettoriali risultano particolarmente rilevanti per modellare i contesti mutati in BRCA1/2, nei quali il signaling dei recettori ormonali è comunemente compromesso.

Nel loro insieme, i risultati di questo lavoro stabiliscono che gli organoidi mammari 3D derivati da iPSC costituiscono una piattaforma robusta e riproducibile, in grado di ricapitolare fedelmente l'organizzazione morfologica, la composizione dei lignaggi cellulari e parziali proprietà funzionali del tessuto mammario umano. I futuri sforzi si concentreranno sull'ottimizzazione del microambiente stromale, sulla riduzione delle sottopopolazioni neurali residue, sul prolungamento del differenziamento verso un fenotipo pienamente secretorio e sull'integrazione dell'editing genico basato su CRISPR per modellare gli effetti delle mutazioni BRCA1/2 in un contesto paziente-specifico. Questo sistema possiede un notevole potenziale traslazionale per applicazioni nella ricerca sul cancro al seno, nello screening dei farmaci e nella medicina oncologica personalizzata.

ABSTRACT (*English*)

The human mammary gland is a complex, hormone-responsive organ whose epithelium is organized into two principal cell populations: luminal alveolar cells responsible for milk secretion, and baso-myoepithelial cells that provide structural support and facilitate milk ejection. These compartments are embedded within a stromal microenvironment that plays a critical role in regulating glandular development and homeostasis. Breast cancer, the most frequently diagnosed malignancy among women worldwide, frequently arises from disruptions in this finely tuned cellular organization, with germline mutations in *BRCA1* and *BRCA2* conferring significantly elevated oncogenic risk. The development of physiologically relevant human *in vitro* models capable of recapitulating mammary tissue architecture is therefore of paramount scientific and clinical importance.

To address the inherent limitations of conventional two-dimensional culture systems, this study presents an optimized multi-step protocol for generating three-dimensional mammary-like organoids from human induced pluripotent stem cells (iPSCs), derived from both healthy female donors and individuals carrying *BRCA1/2* mutations. iPSCs were first aggregated into mammary embryoid bodies (mEBs) using MammoCult medium supplemented with hydrocortisone and heparin, to promote non-neural ectodermal specification. Mammary commitment was subsequently induced with parathyroid hormone-related protein (pTHrP), followed by embedding of 10-day mEBs in a Matrigel/Collagen I composite matrix enriched with FGF10 and HGF to drive branching morphogenesis and alveolar differentiation. Structurally organized organoids were consistently obtained by day 30 of differentiation.

Molecular and histological characterization confirmed successful mammary fate acquisition. Quantitative RT-PCR and immunohistochemical analyses demonstrated progressive downregulation of pluripotency markers (OCT4, NANOG, KLF4) alongside upregulation of luminal markers (GATA3, CK18) and basal/myoepithelial markers (TP63, CK5/6, CK14, SMA), reproducing the characteristic bilayered architecture of the native gland. Residual expression of

neural ectoderm markers PAX6 and FOXG1 was also detected, indicating persistent cellular heterogeneity that remains a challenge for further protocol optimization.

A central finding of this work concerns the dynamic regulation of steroid hormone receptors throughout organoid development. Consistent with the recent demonstration that the progesterone receptor (PR) is constitutively expressed in iPSCs following somatic cell reprogramming — independently of the cell of origin or reprogramming method, and in the absence of estrogen receptor alpha (ER α) — this study tracked PR expression longitudinally across differentiation stages. PR was transiently downregulated during the early mEBs phase and subsequently restored in mature 20-day organoids, mirroring its physiological regulation during mammary morphogenesis. The concomitant expression of PR and GATA3 in mature organoids reflects a functional regulatory axis critical to luminal differentiation and frequently dysregulated in breast cancer. ER α was not detected, in accordance with its known downregulation in actively proliferating mammary epithelium. These receptor dynamics are particularly relevant for modeling *BRCA1/2*-mutant contexts, in which hormone receptor signaling is commonly disrupted.

Collectively, this work establishes iPSC-derived 3D mammary organoids as a robust and reproducible platform faithfully recapitulating the morphological organization, lineage composition, and partial functional properties of human mammary tissue. Future efforts will focus on optimizing the stromal microenvironment, reducing residual neural subpopulations, extending differentiation toward a fully secretory phenotype, and integrating CRISPR-based gene editing to model *BRCA1/2* mutation effects in a patient-specific context. This system holds significant translational potential for applications in breast cancer research, drug screening, and personalized oncological medicine.

CHAPTER 1

Introduction

1.1. Mammary Tissue

Mammary tissue is a complex and dynamic glandular structure primarily responsible for the synthesis and secretion of breast milk. Throughout the many periods of a woman's life, numerous hormonal stimuli that affect its growth and morphological changes are generated.

Unlike most organs, which complete their developmental programme during embryogenesis or shortly thereafter, the mammary gland undergoes the majority of its morphogenesis postnatally, with successive rounds of proliferation, differentiation, and regression occurring during puberty, pregnancy, lactation, and involution. This unique developmental trajectory places the mammary gland at the intersection of endocrinology, developmental biology, and oncology, making it a subject of intense scientific interest (Hassiotou & Geddes, 2013; Lawrence & Lawrence, 2022).

The mammary glands are anatomically situated in the anterior thoracic area, positioned above the pectoralis major muscle, spanning from the second to the sixth rib and from the lateral edge of the sternum to the mid-axillary line. Each breast comprises 15 to 20 glandular lobes, partitioned by connective and adipose tissue, which converge radially at the nipple. Each lobe is splitted into lobules consisting of acini, which discharge into galactophorous ducts; these terminate at the nipple, where they expand into lactiferous sinuses before milk expulsion (Hassiotou & Geddes, 2013; Netter, 2019).

The nipple is encircled by abundant sebaceous glands, while the supportive tissue comprises fibroadipose stroma and suspensory ligaments (Cooper's ligaments) that provide mechanical support to the breast. The vascular supply is chiefly derived from the internal mammary artery, a branch of the subclavian artery, the lateral thoracic artery, a branch of the axillary artery, and intercostal branches (**Figure 1**). The venous drainage parallels the arterial supply, while lymphatic drainage holds significant clinical importance: most lymphatic vessels converge in the axillary lymph nodes, with additional pathways leading to the internal thoracic and supraclavicular lymph nodes (Standring, 2020).

SCHEMATIC ANATOMY OF THE MAMMARY GLAND

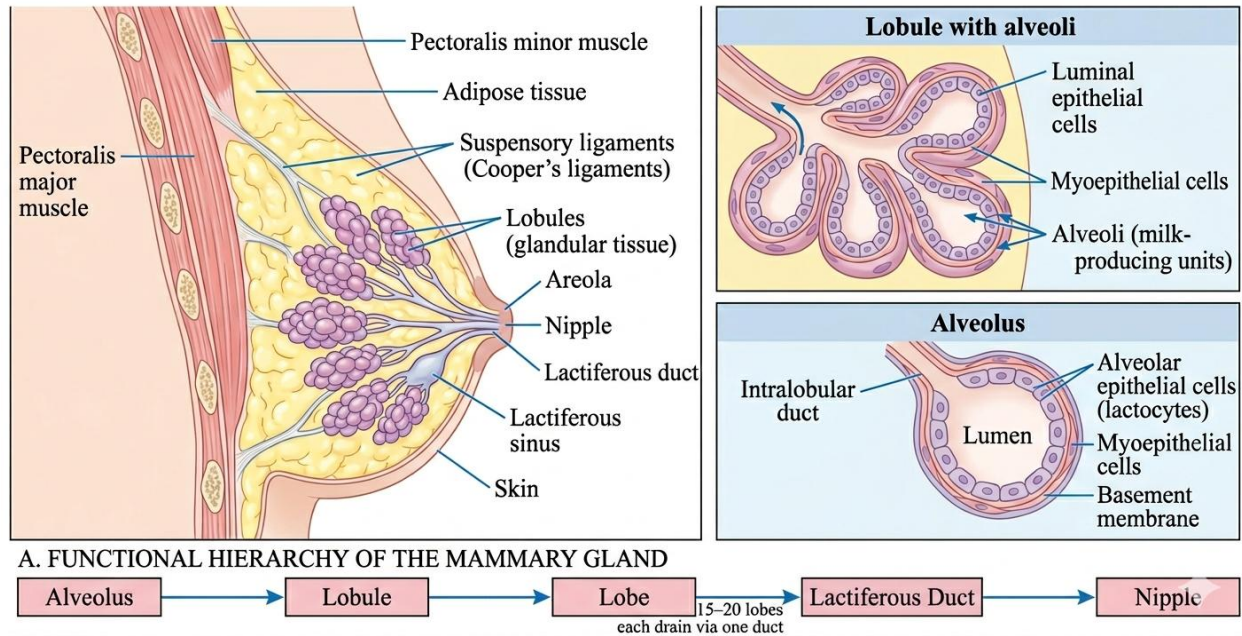


Figure 1: Schematic anatomy of the adult human mammary gland, showing its gross structure, lobular–alveolar microarchitecture, and functional hierarchy from alveolus to nipple.

The principal role of mammary tissue is lactation, which involves the synthesis, production, and expulsion of breast milk. This process is meticulously governed by a sophisticated hormonal system, which is particularly stimulated during pregnancy and the postpartum phase. During puberty, the elevation of estrogen and progesterone stimulates the formation of ducts and glandular lobules. During gestation, alveolar proliferation is further enhanced by elevated levels of estrogen, progesterone, prolactin, placental lactogen, and cortisol. After delivery, the reduction of progesterone and the sustained presence of prolactin facilitate the initiation of milk production, although milk ejection is governed by the reflexive release of oxytocin triggered by suckling (Neville et al., 2002). In addition to this intricate regulatory structure, many hormones, such as insulin, cortisol, and growth hormone, have a role in sustaining secretory activity and cellular metabolism during breastfeeding. The coordinated hormonal signals influence a gland that is not static but continuously evolving: the mammary gland is a dynamic organ whose structure and function are perpetually altered in response to endocrine signals, environmental factors, and physiological conditions throughout the female lifespan (Lawrence & Lawrence, 2022).

1.1.1. The Mammary Glands

The mammary gland is a compound branched tubuloalveolar structure that represents one of the defining characteristics of mammals. From an evolutionary perspective, it derives from two cutaneous glands—the apocrine and epidermal glands—and sits as an accessory bilateral reproductive organ on the ventral surface of the body (Hovey et al., 2002). The primary function of the mammary glands is to produce milk that nourishes offspring after birth, thereby conferring a significant reproductive advantage to mammals (Peaker, 2002).

When we look at its microscopic architecture, the mammary gland is composed of several cell types working in close coordination. The epithelial cells form the ductal network, maximizing the surface area available within a confined space. Meanwhile, a variety of stromal cells—together with extracellular matrix (ECM) proteins—provide essential support. For a visual representation of this supportive arrangement, the reader is referred to (Figure 1B). Among the key components of the stromal connective tissue are adipocytes, which create the mammary fat pad and surround the ductal network; fibroblasts, which help support the hematopoietic system; and vascular endothelial cells, which form blood vessels. The tissue also contains innate immune cells such as macrophages and mast cells, as well as nerve fibers (Muschler & Streuli, 2010; Watson & Khaled, 2008; Biswas et al., 2022).

The adipose stroma serves a threefold purpose: it provides structural support, acts as a supportive network for the bilayered epithelium, and supplies nutrients, blood flow, and immune defenses. It is important to recognize that each specific type of stromal cell releases signals that influence aspects of epithelial development and function.

Within the epithelium, two principal cell lineages exist: luminal and basal. The luminal epithelium lines the inner surface of the ducts (such as the lactiferous duct), surrounds the hollow lumen, and can differentiate into secretory alveoli or lobules that produce milk. In contrast, the basal epithelium consists of myoepithelial cells that form the outer layer of mature mammary ducts; this layer also harbors stem and progenitor cells capable of giving rise to both luminal and myoepithelial cells.

Finally, the epithelium is wrapped by one of the major types of ECM—the basement membrane (BM)—which separates the epithelium from the underlying stroma and actively guides glandular

development. The anatomical relationship between the epithelium, basement membrane, and stroma is illustrated by the terminal end bud (TEB), the bulbous structure at the tip of the growing ductal tree, which drives ductal elongation and branching during puberty (**Figure 2**) (Visvader, 2009).

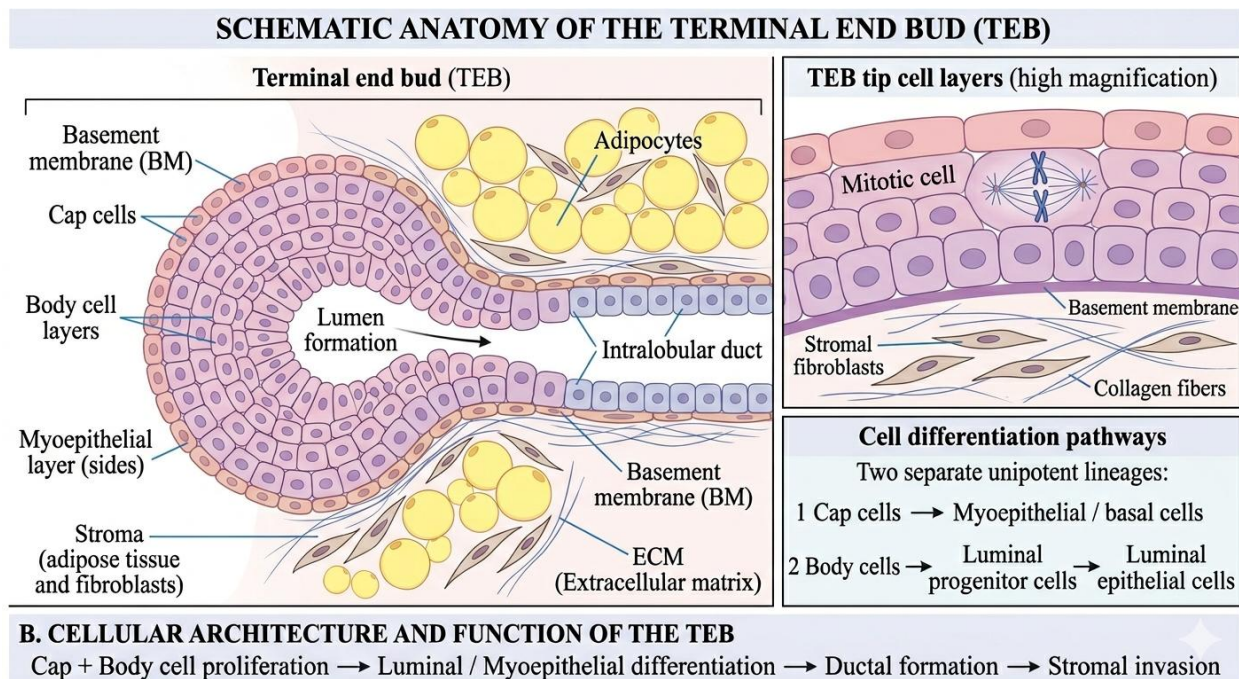


Figure 2: Schematic anatomy of the terminal end bud (TEB), illustrating its cellular organization, lineage differentiation pathways, and role in pubertal ductal morphogenesis.

1.1.2. Structure of the Mammary Gland During Embryogenesis

During embryonic life, the mammary gland begins as a subtle ectodermal thickening in the ventral thoracic region. This initial structure first appears as an epithelial primordium (an epithelial ectodermal bud) that, around the fifth week of gestation (approximately day 35), gives rise to the so-called mammary ridges or “milk lines”. These ridges extend bilaterally from the axilla down to the inguinal region. In this early phase of glandular development, a series of hormone-independent events occur, driven entirely by local signaling between the ectoderm and the underlying mesenchyme (Parmar & Cunha, 2004).

From the sixth week onwards, the ridges gradually regress, becoming confined to two areas in the thoracic region (between the second and sixth ribs). Here, two solid epithelial masses appear and

will invaginate into the underlying mesenchyme. These structures form the primordia of the mammary buds, which are surrounded by fibroblast-like cells embedded within a dense, collagenous connective stroma (**Figure 3**). The process of ridge regression is tightly controlled; any aberration can lead to the development of supernumerary nipples (polythelia) or supernumerary mammary glands (polymastia). These conditions underscore the importance of precise spatial regulation during embryonic mammary development.

Between the seventh and eighth weeks, the mammary parenchyma invades the stroma, producing a prominence called the mammary disc. By the ninth week, the mammary placodes continue to enlarge and assume a conical shape. Between the tenth and twelfth weeks, epithelial buds emerge from these placodes and transform into globular structures, thereby defining the boundary between the epithelial and stromal components. The subsequent branching stage of the parenchyma extends from the thirteenth to the twentieth week, culminating around the fifteenth week in the formation of 15 to 25 solid epithelial cords, which will eventually give rise to the ductal system of the mature gland.

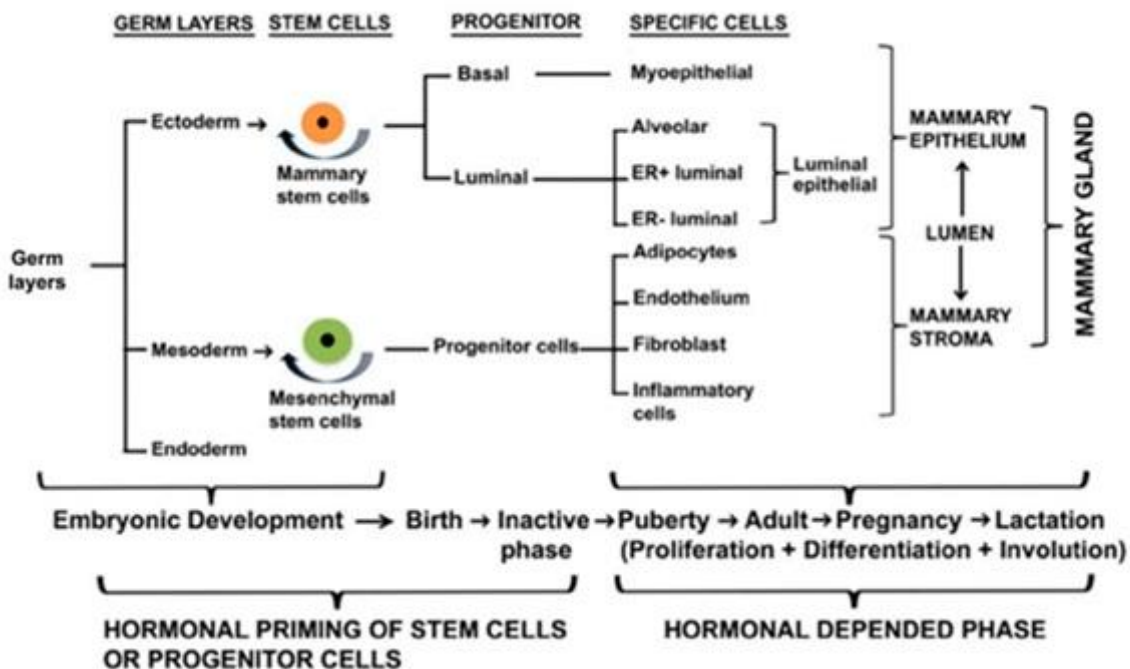


Figure 3: Schematic representation of the origin of epithelial and mesenchymal cells and the prenatal development of the mammary gland from embryonic to adult stage (Biswas S.K. et al., 2022).

During the second trimester (specifically between the twelfth and sixteenth weeks), the nipple and areola begin to differentiate. Meanwhile, the surrounding mesenchymal cells give rise to fibroblasts, smooth muscle cells, endothelial cells, and adipocytes. By the twentieth week, canalization of the epithelial cords into primitive ducts is observed, while the cutaneous region of the nipple invaginates to form the mammary pit. At this stage, the epithelial cells organize into a bilayer: the inner (luminal) layer progressively acquires secretory characteristics, and the basal layer gives rise to myoepithelial cells (**Figure 3**) (Parmar & Cunha, 2004).

By the sixth month of gestation, the basic tubular architecture of the gland is established. At 32 weeks of gestation (Hovey et al., 2002), the primary lactiferous ducts open into the area that will develop into the nipple (Javed & Lteif, 2013). The adipose tissue of the mammary gland loses its ability to form fibers and to support further parenchymal growth; instead, it begins to form adipose islands surrounded by fibrous stroma (Vorherr, 1974). The primitive secretory epithelial cells, which become functional towards the end of gestation, respond to the lactogenic hormones of pregnancy (Parmar & Cunha, 2004). In the neonate, the mammary glands are very rudimentary: the ducts terminate in short ductules, or epidermal ridges lined by one to two layers of epithelial cells and a single layer of myoepithelial cells. Shortly after birth, these epithelial cells produce colostrum—a phenomenon attributable to circulating lactogenic hormones in the newborn. This secretory activity diminishes within three to four weeks of life (Russo, 1987).

The basement membrane of the mammary bud and its early projections contain type IV and type VII collagen as well as laminin- $\alpha 3$, while the overlying epithelium expresses integrins $\beta 1$, $\beta 4$, and $\alpha 6$. However, the precise function of the basement membrane in embryonic gland development remains poorly understood (Jolicœur, 2005). Regression of the mammary gland typically occurs within four weeks after birth, coinciding with a decrease in prolactin secretion from the neonatal anterior pituitary gland (Vorherr, 1974). Following this regression, the mammary gland becomes quiescent until the onset of puberty; this quiescent state is manifested by ductal regression shortly after birth.

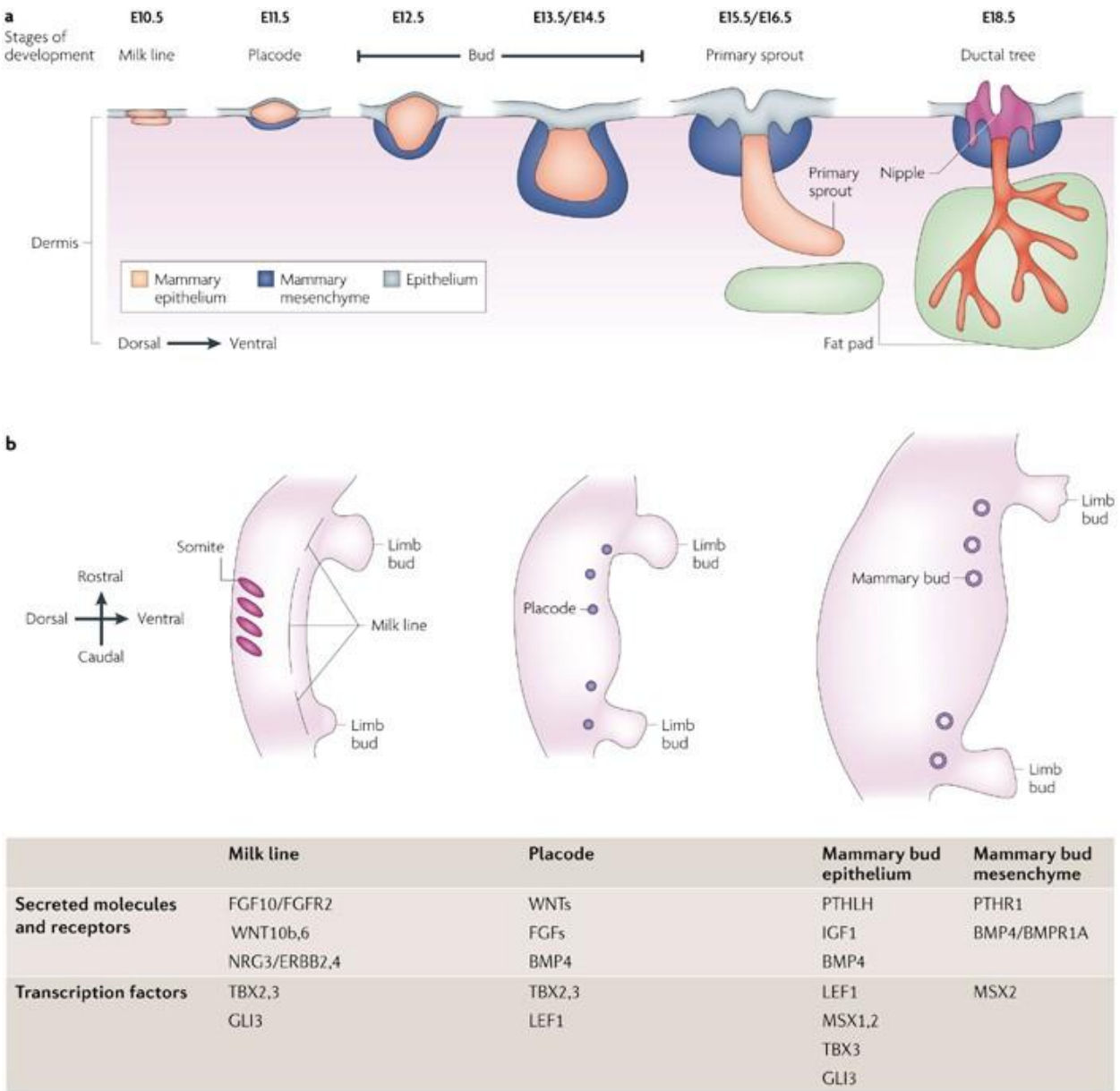
1.1.3. Signaling Pathways Involved During Embryogenesis

Embryonic mammary development is hormone-independent: the receptors for growth hormone (GHR), estrogen (ER α and ER β), prolactin (PRLR), and progesterone (PR) are dispensable for bud formation (Hassiotou & Geddes, 2013; Hennighausen & Robinson, 2001). Morphogenesis is instead driven by a reciprocal dialogue between the mesenchyme and the overlying ectoderm, orchestrated by a conserved network of signaling families—Wnt/ β -catenin, fibroblast growth factor (FGF), Hedgehog (Hh), insulin-like growth factor 1 (IGF-1), parathyroid hormone-related protein (PTHrP), and neuregulin-3 (NRG3)—that together remodel the ectoderm, expand the mammary line and promote placode assembly and bud outgrowth (Hens & Wysolmerski, 2005) (**Figure 4**).

Wnt/ β -catenin signaling is central: Wnt ligands engage Frizzled and LRP5/6, stabilizing β -catenin and activating target-gene transcription, and the pathway is indispensable for placode specification, as overexpression of the antagonist DKK1 abolishes mammary morphogenesis (N. Ebrahimi et al., 2022; Chu et al., 20). mammary line and promote placode assembly and bud outgrowth (Hens & Wysolmerski, 2005) (Figure4)04). In parallel, FGF10 expressed along the mammary line and its receptor FGFR2 β in the epithelial placodes are essential for bud specification and outgrowth (Mailleux et al., 2002; Veltmaat et al., 2006). IGF-1 provides a complementary systemic input through Rho-GTPase-activating proteins and IRS1/2, linking somatic growth to glandular development (Kleinberg et al., 2009), while the strictly local PTHrP/PTH1R axis—epithelial PTHrP sensitizing the PTH1R-expressing mesenchyme—supports ductal, nipple, and nipple-sheath formation and is required for bud formation (Hens et al., 2007; Neville et al., 2002). Hh signaling and NRG3, acting via ErbB4, further refine mammary cell fate.

These pathways converge on transcription factors of the HOX, GATA, and T-box families (Hens & Wysolmerski, 2005). TBX3 is particularly important: its haploinsufficiency in humans causes

ulnar–mammary syndrome with severe mammary hypoplasia or agenesis. Induced by Wnt and FGF, TBX3 reinforces both pathways in a positive-feedback loop that drives the transition from



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Figure 4: Cooperation of signaling pathways in embryonic mammary gland development (Gertraud W. Robinson Ramadas, 2017).

mammary line to placode, whereas BMP4 antagonizes this circuit by repressing TBX3; HOXC8 and HOX9 provide additional spatial patterning (Bamshad et al., 1997; Chu et al., 2004; Hynes & Watson, 2010; Pond et al., 2013). Remodeling also involves macrophages, which populate the peri-

bud mesenchyme and partially dismantle the epithelium in male embryos—contributing to sexual dimorphism—before relocating postnatally to intraductal niches and establishing a persistent immune-surveillance network (Dawson et al., 2020; Stewart et al., 2019). Underlying these events is the regulation of the epithelial–mesenchymal transition (EMT) and its reverse (MET), through which epithelial cells alternately acquire and lose migratory traits. Both transitions are integral to normal morphogenesis, yet the same programme is co-opted in breast cancer, where tumor-initiating cells acquire stem-like properties via EMT (Creighton et al., 2009; Ye et al., 2015)—a parallel that predicts the mechanistic continuum between mammary development and tumor biology explored in the sections that follow.

1.1.4. Structure of the Mammary Gland During Puberty

The mammary gland goes into morphogenetic quiescence after birth. However, until adolescence, The primitive mammary structure shows the same growth rate as the rest of the body. At this stage, the developing female and male breast are indistinguishable. (Sternlicht, 2005) (**Figure 5**).

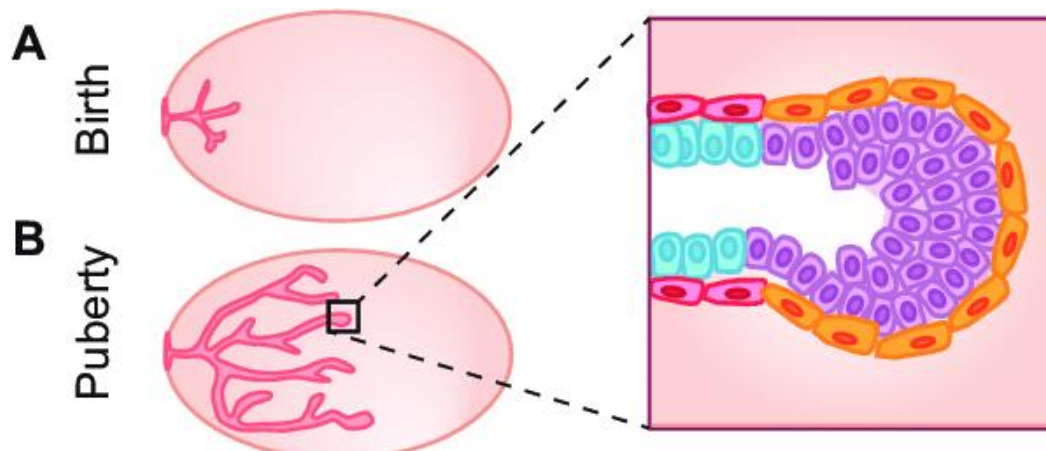


Figure 5: gland after birth and during puberty in women (Hannan F.M. et al., 2023).

In males, the onset of puberty triggers androgen-mediated mesenchymal condensation around the primary ducts, leading to duct elimination and the suppression of mammary epithelial growth, which renders the male breast non-functional (Dunbar et al., 1999). In females, puberty—typically beginning between 8 and 12 years of age—is marked by the production of female sex hormones (primarily estradiol, E2, and progesterone, P4) together with pituitary growth hormone (GH) and other systemic factors. These signals promote a series of coordinated events that transform the rudimentary mammary structure into a fully functional gland. Throughout reproductive life, the

female mammary gland undergoes changes closely linked to the ovarian and uterine cycles (Russo & Russo, 2004).

The glandular pattern during pubertal development is characterized by long ducts with intermittent branching points, interspersed with niches containing progenitor cells. These niches are organized by integrin matrix interactions, which in turn are regulated by lateral inhibition signals and planar cell polarity mechanisms. Ductal elongation is facilitated by cadherin expression and cytoskeletal contraction, while branching is regulated by processes driven by growth factors, hormones, extracellular matrix (ECM) components, matrix metalloproteinases (MMPs), and immune cells (Brennan & Brown, 2004; Lu & Werb, 2008; Nishimura & Takeichi, 2009; Visvader, 2009).

The most critical structures during pubertal development are the terminal end buds (TEBs), which are unique to the peripubertal period and serve as the primary drivers of ductal expansion. From the TEBs, collagen fibers radiate outward into the ECM, providing structural guidance for ductal penetration into the fat pad. During puberty, the ductal tree becomes progressively more complex: primary ducts branch into segmental and subsegmental ducts, ultimately reaching the terminal portion where terminal ductules form and culminate in acini (Howard & Gusterson, 2000). This cluster of acini branching from a single terminal duct is defined as the terminal ductal lobular unit (TDLU) and is enclosed within loose connective tissue (**Figure 6**). Each TDLU exists at a different stage of lobular differentiation, classified from type I to type IV based on morphological complexity (Russo & Russo, 1987). TDLUs are of considerable clinical importance, as they represent one of the principal sites from which breast carcinomas originate (Wellings & Jensen, 1973).

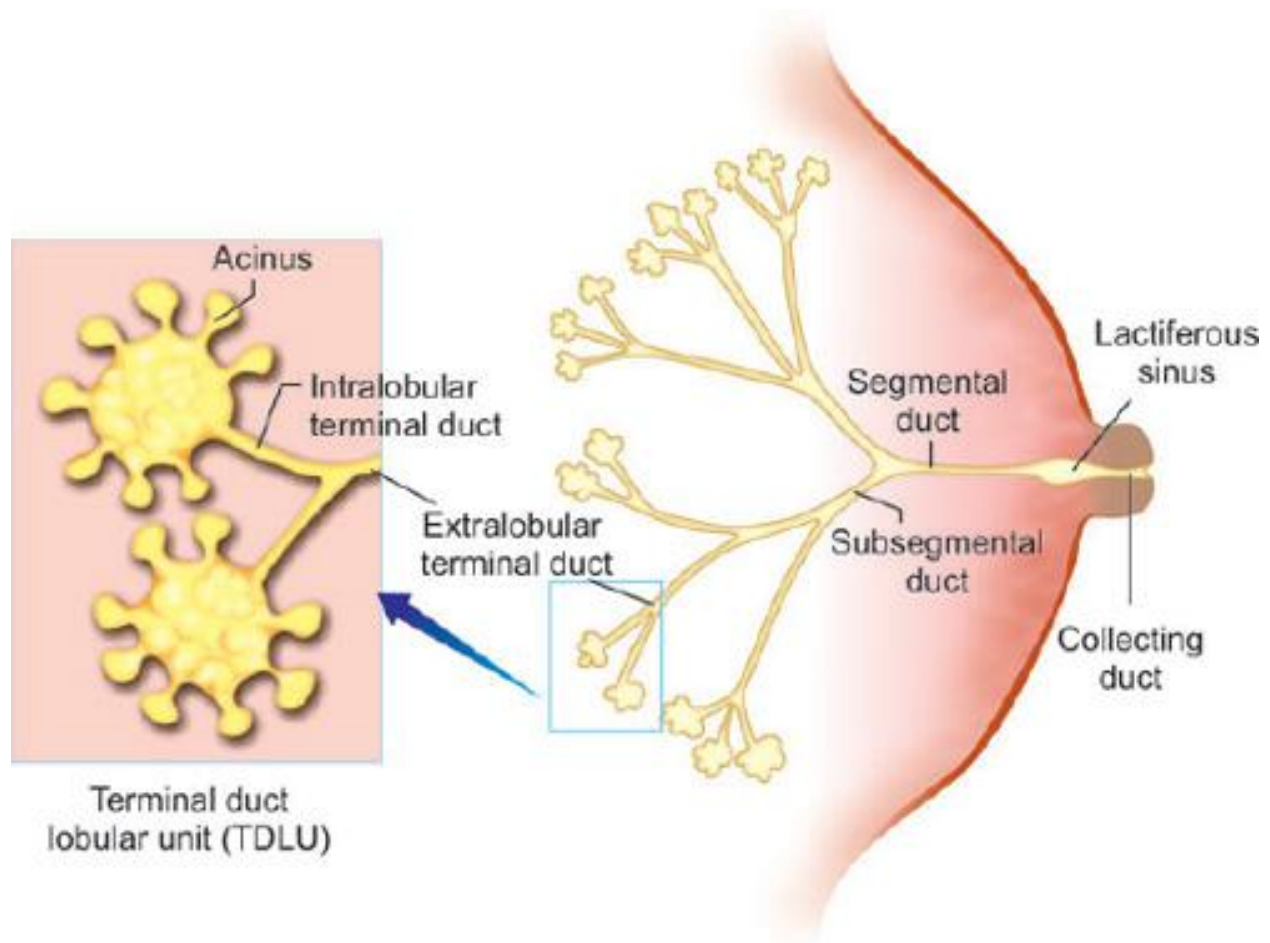


Figure 6: Breast anatomy: lobes, lobules (TDLU) and ducts (Nayak Ramadas, 2017).

In addition to the involvement of stroma and epithelium in allometric growth, there is also an increase in adipose tissue deposition. Regarding ductal development, the initiation of ductal growth is termed “anlage” (denoting the earliest developmental stages) and proceeds through a process defined as “patterning”, in which specific mechanisms for the elongation and branching of thin tubes toward the pre-existing mammary stromal fat pad are activated. The terminal tips extend to the margins of the mammary fat pad and subsequently regress, leaving truncated branches. Terminal end buds are highly proliferative, hormone-dependent structures that serve as the engines of ductal morphogenesis; they are unique to the peripubertal period (**Figure 7**).

At the structural level, TEBs are divided into two distinct cellular compartments. The first compartment consists of cap cells, which form the outer layer as a single proliferative cell layer localized on the distal surface. Cap cells produce the basal lamina and give rise to the myoepithelial cells of mature ducts. They possess characteristics similar to stem cells and can indeed give rise to

both luminal and myoepithelial cells. Studies on cells expressing the P63 protein have identified specific cap cells with unipotent differentiation capacity toward a myoepithelial lineage, supporting the hypothesis of a precise hierarchy in mammary cell differentiation (Sreekumar et al., 2017).

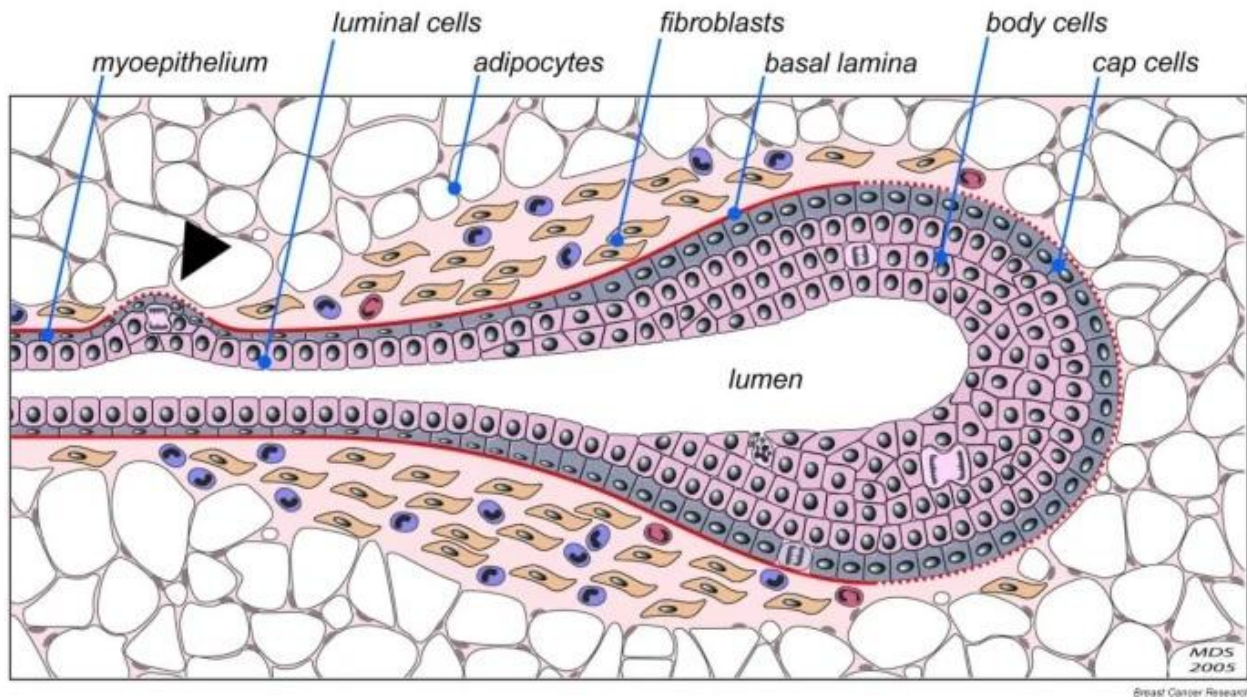


Figure 7: Structure of Terminal End Buds (TEBs) (Mark D Sternlicht, 2005).

The second compartment consists of body cells, which form the inner layer of TEBs and give rise to the luminal cells that line the ducts, functioning as ductal and alveolar progenitors. The most central body cells are weakly cohesive via desmosomes and partially polarized, whereas those closest to the basal lamina are polarized and connected by adherens junctions. During lumen formation, this layer exhibits an increased rate of apoptosis relative to cap cells; indeed, BIM proteins of the BCL2 (BH3-only) family and FOXO1 are activated, triggering apoptosis and cell removal to create the hollow ductal lumen (Mailleux et al., 2007). Overall, TEBs are composed of a pool of plastic, heterogeneous cells that drive ductal expansion during puberty.

Ductal elongation is also mediated by communication between mammary epithelial cells (MECs) and the stroma, as well as by certain immune cells. Following the activation of specific factors, eosinophils are recruited to orchestrate lateral branching, while macrophages surround TEBs and

assist in ductal growth into the fat pad and in the phagocytosis of body cells during duct formation (Gouon-Evans et al., 2000; Ingman et al., 2006; Stewart et al., 2019).

The role of the ECM is of great importance in supporting stroma–epithelium interactions and patterning during glandular morphogenesis. The more complex its structure, the greater the organization of TEBs and lateral branches. The mechanical properties of the ECM support stroma–epithelium interactions through numerous weak non-covalent bonds and covalent cross-links that organize the scaffold, providing structural tension and stability. ECM accumulates in the cleft between bifurcating end buds, facilitating divergent growth and thereby influencing the pattern of ductal branching. Depending on ECM density and composition, the fate of epithelial cells is determined: adhesion, survival or death, polarity, proliferation, and differentiation of stem and progenitor cells are all influenced by the physical and chemical properties of the surrounding matrix (Butcher et al., 2009; Fata et al., 2004; Guilak et al., 2009; Maller et al., 2010).

1.1.5. Signaling Pathways Involved During Puberty

At the onset of puberty, the mammary gland presents an early structure that is asymmetrically branched as a result of the hormone-independent processes that occurred during embryogenesis. However, during puberty, the hormones of the hypothalamic-pituitary-ovarian axis strongly influence the elongation and branching of the mammary ducts. These hormonal signals diffuse into the terminal end buds (TEBs), the epithelium, and the stroma, leading to changes that are further amplified by autocrine and paracrine signals within the gland itself.

The first morphological changes become evident with the onset of the menstrual cycle. With each cycle, the rudimentary gland is progressively transformed into a complex ductal network through TEB bifurcation, lateral secondary branching, and the formation of tubuloalveolar structures. During the follicular phase, lobules are relatively quiescent—small, with few alveoli and low mitotic activity. In the luteal phase, following ovulation, lobules and alveoli develop open lumina and exhibit maximal mitotic activity. Several mechanisms regulate the timing of end-bud bifurcation and, more generally, the elaboration of the ductal network; indeed, during menstrual cycles and pregnancy, lateral branches and alveoli appear and disappear intermittently.

Among the principal transcription factors participating in mammary ductal morphogenesis, the most important are GATA3 and AP-2 γ , because they guide the genetic programmed activated by

ovarian hormones (Jäger et al., 2010). Additional morphogenetic modifications occur not only through steroid hormones and growth factors but also through processes such as progenitor cell proliferation, differentiation, tissue homeostasis, and apoptosis.

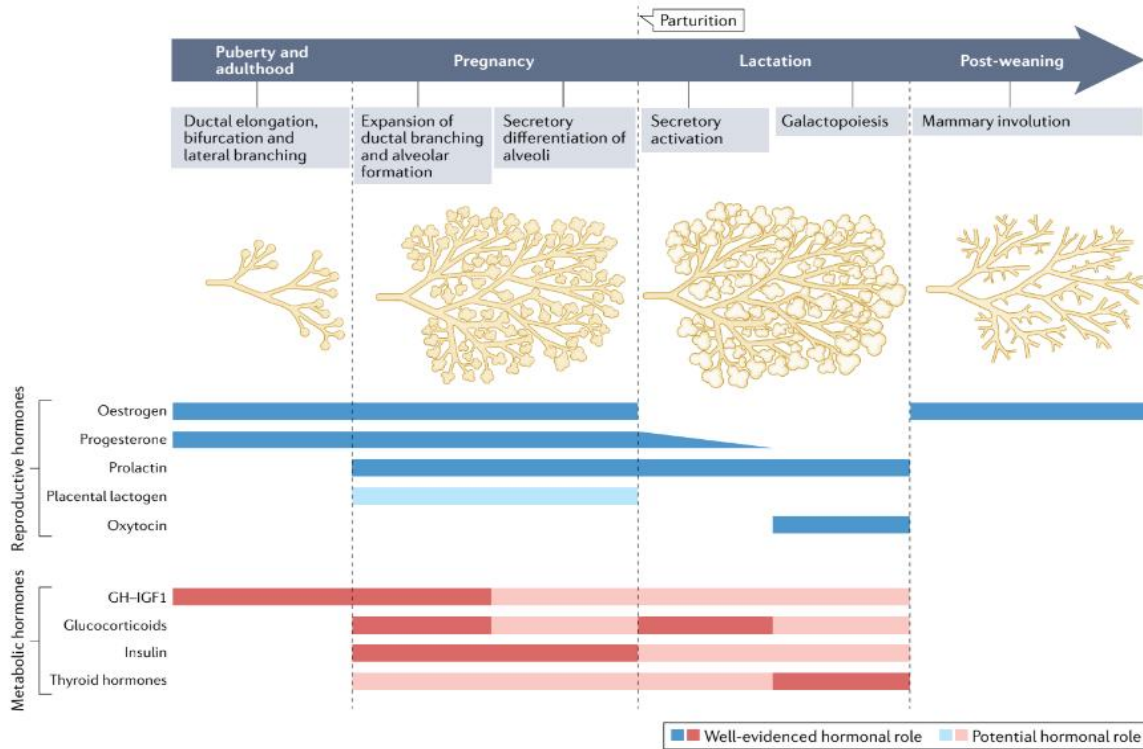


Figure 8: hormones that influence mammary gland development during the developmental stages of women (Hannan F.M. et al., 2023).

Estrogens, the principal female sex hormones, play a key role in ductal branching of the mammary gland, regulating epithelial proliferation through coordinated action with local growth factors and via interactions between stromal and epithelial cells. During puberty, estrogen is secreted by ovarian follicles during the phases of the menstrual cycle and is also synthesized directly by mammary adipose tissue through the enzyme aromatase. From the stroma, various paracrine signals lead to the activation of estrogen receptors (ER), which are primarily present on luminal cells (**Figure 8**).

The expression of the estrogen receptor gene is regulated by multiple elements: transcription factors, paracrine, endocrine, and autocrine factors, as well as environmental influences. The presence of ER is fundamental for the development of TEBs, the ducts of the fat pad, alveologenesis, and lactation during pregnancy. Notably, ER presence in the stroma is not required

for glandular development, as demonstrated by tissue recombination experiments in which ER-null stroma combined with wild-type epithelium supported normal mammary development. Estrogen (E2) also stimulates communication between endothelial cells, adipocytes, and the epithelium, establishing a multicellular dialogue that coordinates glandular morphogenesis.

Another important transcription factor, Forkhead box A1 (FOXA1), mediates E2 signaling by facilitating chromatin accessibility and thereby enabling the interaction between ER and its target genes. FOXA1 has been identified as a target of the GATA3 regulatory network in branching morphogenesis during puberty. Loss of GATA3 abolishes TEB formation and reduces ductal growth, demonstrating the essential role of this transcription factor in mammary development (Hurtado et al., 2011; Kouros-Mehr et al., 2006).

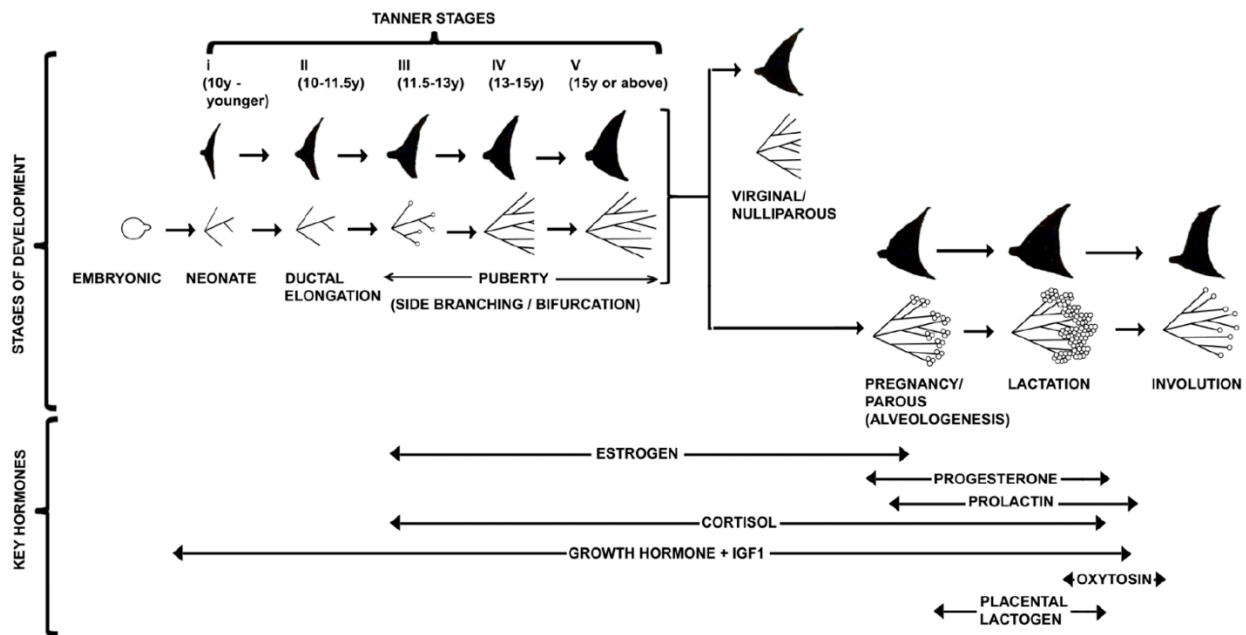


Figure 9: Schematic representation of the different stages of mammary gland development associated with the influence of hormones (Biswas S.K. et al., 2022).

In ER-positive luminal cells of TEBs and mature ducts (**Figure 2**), progesterone receptors (PR) are specifically expressed, particularly during puberty owing to the rise in estrogen levels. Together with E2 and IGF-1, progesterone (P4) is implicated in TEB expansion and lateral branching rather than in ductal elongation and extension (Fata et al., 2001). P4 is of considerable importance because it acts through paracrine signals, leading to the activation of factors such as RANKL (receptor activator of nuclear factor kappa-B ligand), Wnt4, and EGFR (epidermal growth factor receptor), thereby contributing to the proliferation of mammary stem cells (MaSCs) that lack PR

expression (**Figure 11**). This paracrine mechanism is essential because enables PR-expressing luminal cells to communicate proliferative signals to PR-negative stem and progenitor cells, thereby expanding the stem-cell pool without requiring direct hormone sensing (Pal et al., 2013; Schams et al., 2003; Shehata et al., 2012; Shyamala et al., 2002).

IGF-1 signaling represents another important paracrine pathway that is essential for mammary gland growth and development. IGF-1 mediates the effect of growth hormone (GH) and is synthesized primarily in mammary stromal fibroblasts, acting on epithelial cells that express the IGF-1 receptor (IGF-R). Its action also regulates the responsiveness of epithelial cells to E2 and P4, establishing a hierarchical signaling network in which systemic and local hormonal signals converge on the mammary epithelium. Development is further potentiated by E2 in the stroma–epithelium interaction (**Figure 9**) (Kleinberg et al., 2000; Kleinberg & Ruan, 2008; Macias & Hinck, 2012; Rusidzé et al., 2021; Zeps et al., 1998).

1.1.6. Development of the Mammary Gland During the Gestational Phase

At the onset of the gestational phase, the morphological maturation of the mammary gland is completed. Under the influence of progesterone, prolactin, and other systemic factors, extensive tissue remodeling occurs to mediate epithelial expansion and the formation of secretory structures (Bocchinfuso et al., 2000; Cowin & Wysolmerski, 2010; Das & Vonderhaar, 1997; Ormandy et al., 1997). The process is biphasic: modifications in alveolar growth occur concomitantly with tertiary branching.

In the first phase following conception, there is an increase in mammary epithelial cell (MEC) proliferation to stabilize alveolar progenitor cells, which will subsequently undergo differentiation (Russo et al., 2005; Russo & Russo, 2004).

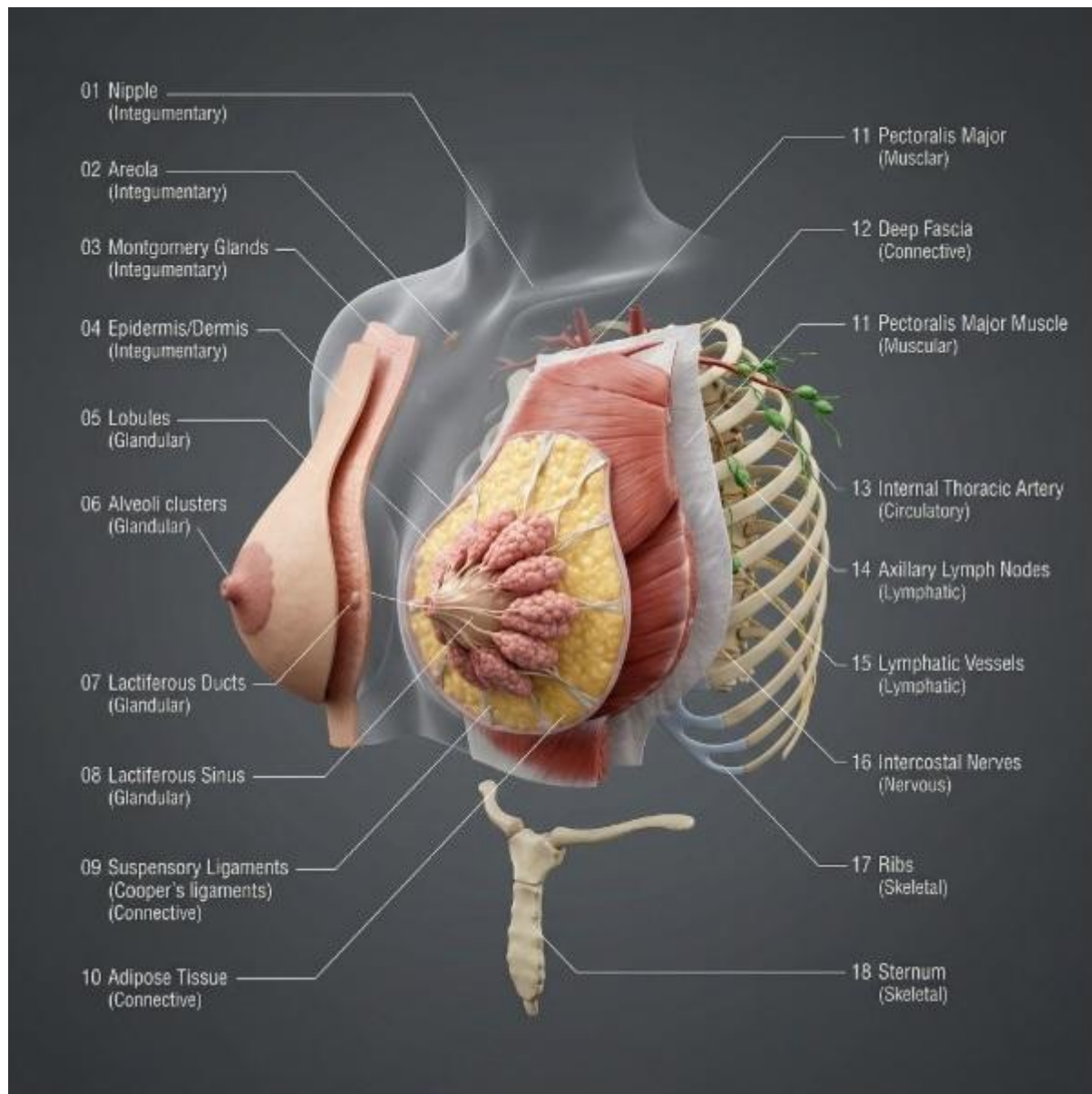


Figure 10: Breast anatomy (bodypartarchive, 2025).

As pregnancy progresses, and particularly in its final stages, progenitor cells differentiate into mature alveolar cells. Their activation during pregnancy is fundamental for the formation of secretory Alveoli. It has been hypothesized that mature alveolar cells derive from unipotent luminal progenitors that developed during embryogenesis from embryonic mammary cells. After the postnatal period, these cells remain quiescent, whereas during gestation they are reactivated and differentiate into specialized alveolar cells. Within the embryological context, it has also been

hypothesized that these stem cells possess multilineage potential, although their precise origin and role remain poorly elucidated.

Structurally, the alveoli present a spherical morphology containing a single layer of secretory epithelial cells connected by tight junctions that produce the components of milk. Externally, a layer of myoepithelial cells is present, covered by a basement membrane (Hynes & Naba, 2012). In support of alveolar development, there is an increase in the number of cytokeratins (cytoskeletal molecules that provide support to epithelial cells) and claudins (which form tight junctions that confer cohesion between epithelial cells). The latter are of great importance because, towards the end of pregnancy, under the stimulus of prolactin, they close to seal the alveolar epithelium, thereby maintaining the composition of milk without dispersal of molecules toward the stroma (Blackman et al., 2005; Rudolph et al., 2003).

The final phase of glandular differentiation is completed after the 22nd week of pregnancy and is directly correlated with the concentrations of human placental lactogen. By mid-pregnancy, epithelial proliferation diminishes; ductules begin to progress toward the formation of secretory acini, lipid accumulation occurs, and stromal remodeling takes place. Remodeling leads to a more vascularized stroma in which immune cell infiltration occurs, and nutrients are supplied to alveolar structures through the loss of lipid droplets from adipocytes (Briskin et al., 2002; D'Cruz et al., 2002).

All these changes serve to prepare the gland for milk production. Before the lactation phase, the gland is composed of glandular tissue, adipose tissue, and fibrous connective tissue known as Cooper's ligaments. The lobules constitute glandular tissue and are, in turn, formed by alveoli that are drained by ducts. There are approximately 15 to 20 ducts that converge into larger ducts until reaching the central lactiferous duct, localized in the nipple (**Figure 10**) (Hartmann, 1991; Love & Barsky, 2004; Moffat & Going, 1996). The terminal lobular units serve as sites of colostrum accumulation and facilitate the formation of lobular secretory alveoli (Hennighausen, 2001). The consequent increase in the number and size of lobules leads to a replacement of adipocytes and a reduction in stromal volume. The high rate of cellular proliferation declines shortly before delivery (Atabai et al., 2007; Lester, 2005; Neville et al., 1998; Traurig, 1967).

Beyond the internal tissue changes, notable modifications also occur in the periareolar area. Among the first visible signs of pregnancy is an increase in the size and number of sebaceous

glands on the areola, known as Montgomery's glands. These glands undergo changes even before breastfeeding begins, secreting an oily substance that protects and lubricates the nipple during suckling.

The process of milk production begins at the alveoli, where basal and luminal cells originate from alveolar precursors; the latter further differentiate into milk-producing cells (Hennighausen & Robinson, 2005). The cellular modifications described above lead to an overall transformation of the glandular architecture, with the formation of specialized structures dedicated to the secretion and transport of milk. The most critical phase is the process of alveologenesis, which ends with the formation of milk-secreting acini, the expansion of the alveolar epithelium, tertiary branching, and tertiary tissue invasion. Following differentiation, the number of lobules ranges between 20 and 40, and the gland presents branched ducts and tree-like epithelial structures.

1.1.7. Hormonal Pathways Activated During Gestation

The hormones that play a leading role during pregnancy differ substantially from those described during puberty. During pregnancy, progesterone and prolactin take on paramount importance for tubuloalveolar development, whereas estrogen primarily mediated ductal development during puberty. The sequential development mediated by estrogen during puberty is fundamental for ensuring that the various morphological stages occur in the correct order and that alveolar buds can form properly to transport secretions efficiently.

In women, progesterone (P4) is produced by the corpus luteum following ovulation and by the placenta during pregnancy. The expression of the progesterone receptor (PR) in the luminal mammary epithelium is important because it mediates cellular proliferation and establishes the spatial organization of how alveolar progenitors will be arranged. Progesterone works synergistically to support cellular proliferation and alveolar maturation by activating several paracrine factors, among which the most important are IGF-1, E2, RANKL, STAT5, and PRLR. Wnt and RANKL are particularly important because they mediate the paracrine induction of β -catenin in basal cells, promoting lateral branching and alveologenesis (**Figure 11**).

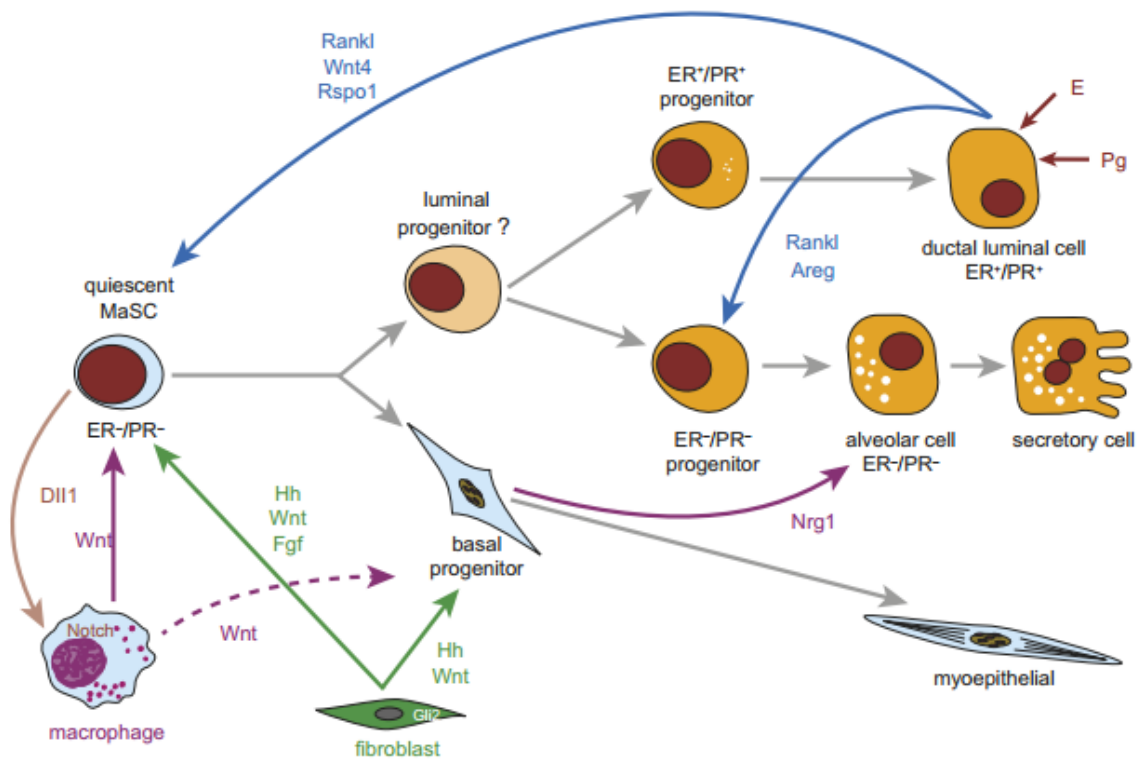


Figure 11: Interactions between epithelial cells, stem cells, and the stromal environment in regulating mammary duct development (Fu et al., 2020).

Prolactin (PRL) production is of great importance during this phase. Prolactin is synthesized in the pituitary gland and is fundamental because it supports the secretion of E2 and P4 from the corpus luteum and the placenta, thereby indirectly acting on alveolar proliferation and functional differentiation of mammary epithelial cells (MECs). After the midpoint of pregnancy, these functions are increasingly performed by placental lactogens. Prolactin is important not only during alveologenesis but also during the differentiation of MECs into milk-secreting cells; indeed, following pregnancy, with the decline of progesterone, it activates milk secretion. Through dimerization of the PRLR receptor, the action of two key transcription factors is activated: STAT5 regulates the expression of milk genes, which encode proteins that compose milk, such as β -casein and whey acidic protein (WAP); and ErbB4 supports the lactogenic differentiation already mediated by STAT5 (Miliku & Azad, 2018).

The proliferation of lobuloalveolar structures is supported at the beginning of pregnancy by the inhibitor of differentiation, which mediates the invasion, migration, and survival of mammary epithelial cells. The extracellular matrix (ECM) also plays an important role during pregnancy and

lactation, as it is produced during these phases of lobuloalveolar differentiation and influences the traction forces of epithelial cells. For the correct formation of the ductal lumen, Bcl-2-dependent apoptosis is also required, in this context, the ECM remains fundamental as it guides the orientation of luminal cells and the creation of cavities. Another crucial hormone is placental lactogen, which is secreted by the placenta and is correlated with glandular growth, as it stimulates the activation of the prolactin receptor (Miliku & Azad, 2018).

1.1.8. Morphology of the Mammary Gland During Lactation

During lactation, the mammary glands release milk and to support this production numerous blood vessels are present within the stroma, conveying a wide range of micro and macromolecules (Djonov et al., 2001). The structure of the gland during this phase is specifically adapted for collecting milk within the lobes and then expelling it toward the ducts that connected to the nipple (Figure 12).

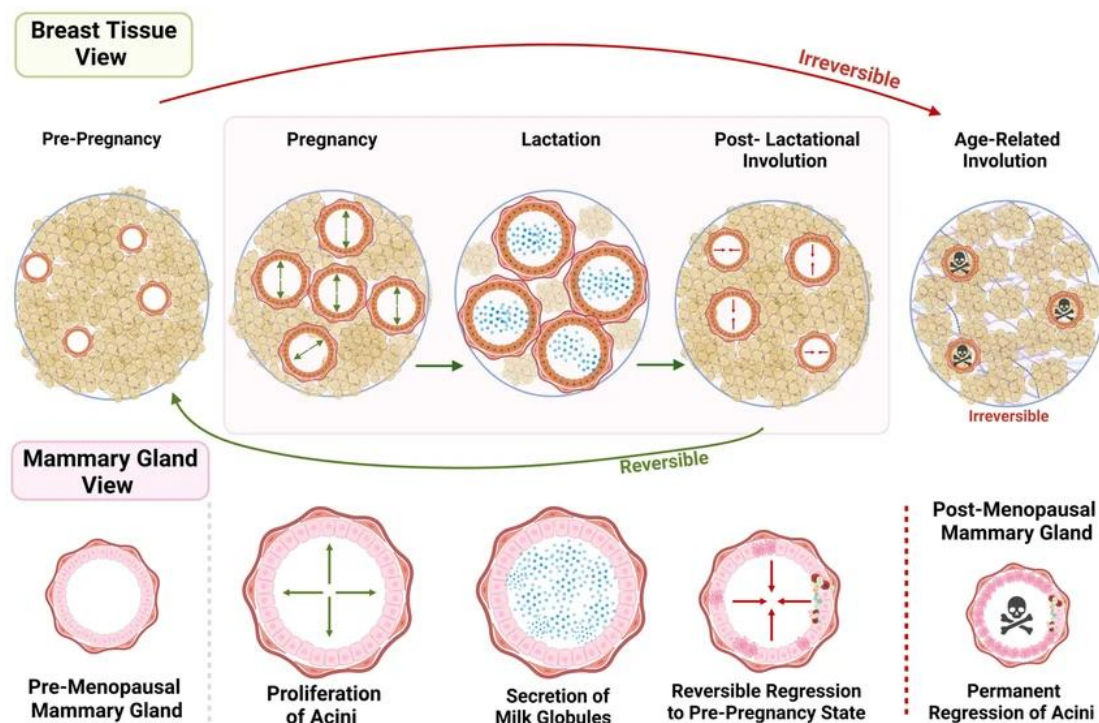


Figure 12: evolution of the mammary gland pre pregnancy till age-related (Jaida C. Lue, 2025).

Inside the alveoli, luminal epithelial cells secrete milk. Under the stimulus of oxytocin, the myoepithelial cells that surround the alveoli contract in a synchronized and coordinated manner, enabling the expulsion of milk into the lactiferous ducts. This contractile response is possible because myoepithelial cells are electrically coupled through gap junctions, allowing rapid and coordinated contraction across the entire gland. During this phase, oxytocin is highly expressed compared to progesterone, which was elevated during pregnancy to inhibit premature milk production (Howard & Gusterson, 2000). Oxytocin also plays an important role in regulating calcium absorption during lactation (Moore et al., 1988).

Another hormone of vital importance during the final stages of pregnancy and lactation is cortisol. In mammary epithelial cells, cortisol promotes the formation of rough endoplasmic reticulum, where milk proteins are synthesized; it also helps regulate estrogen (E2) activity and the production of late milk proteins.

1.1.9. Hormones Involved in the Maintenance of Lactation

During delivery, a rapid reduction in progesterone occurs to facilitate the phase of secretory activation, which was inhibited throughout the gestational period. After delivery, with the removal of the placenta, circulating progesterone levels fall markedly, while plasma concentrations of prolactin remain high (Pang & Hartmann, 2007).

Lactation is sustained by both systemic hormones and local regulatory factors. Primarily, through suckling, olfactory and auditory stimuli from the neonate trigger neuroendocrine signals that lead to prolactin secretion from the anterior and posterior pituitary gland (Boss et al., 2018). Shortly before suckling, external stimuli such as the infant's cry facilitate the gradual release of oxytocin, which mediates milk ejection and increases intracellular calcium levels in myoepithelial cells. This, in turn, causes a synchronized contraction of the alveoli and ducts, delivering milk to the nipple. Lipid droplets are also released by luminal mammary epithelial cells into the alveolar lumen. **(Figure 13).**

Prolactin levels are highest during the first months of breastfeeding and subsequently decline progressively. Importantly, the quantity of prolactin is not correlated with the rate of milk synthesis; rather, milk production is positively associated with the degree of breast emptying. This finding suggests that milk synthesis is regulated locally by the mammary gland itself through

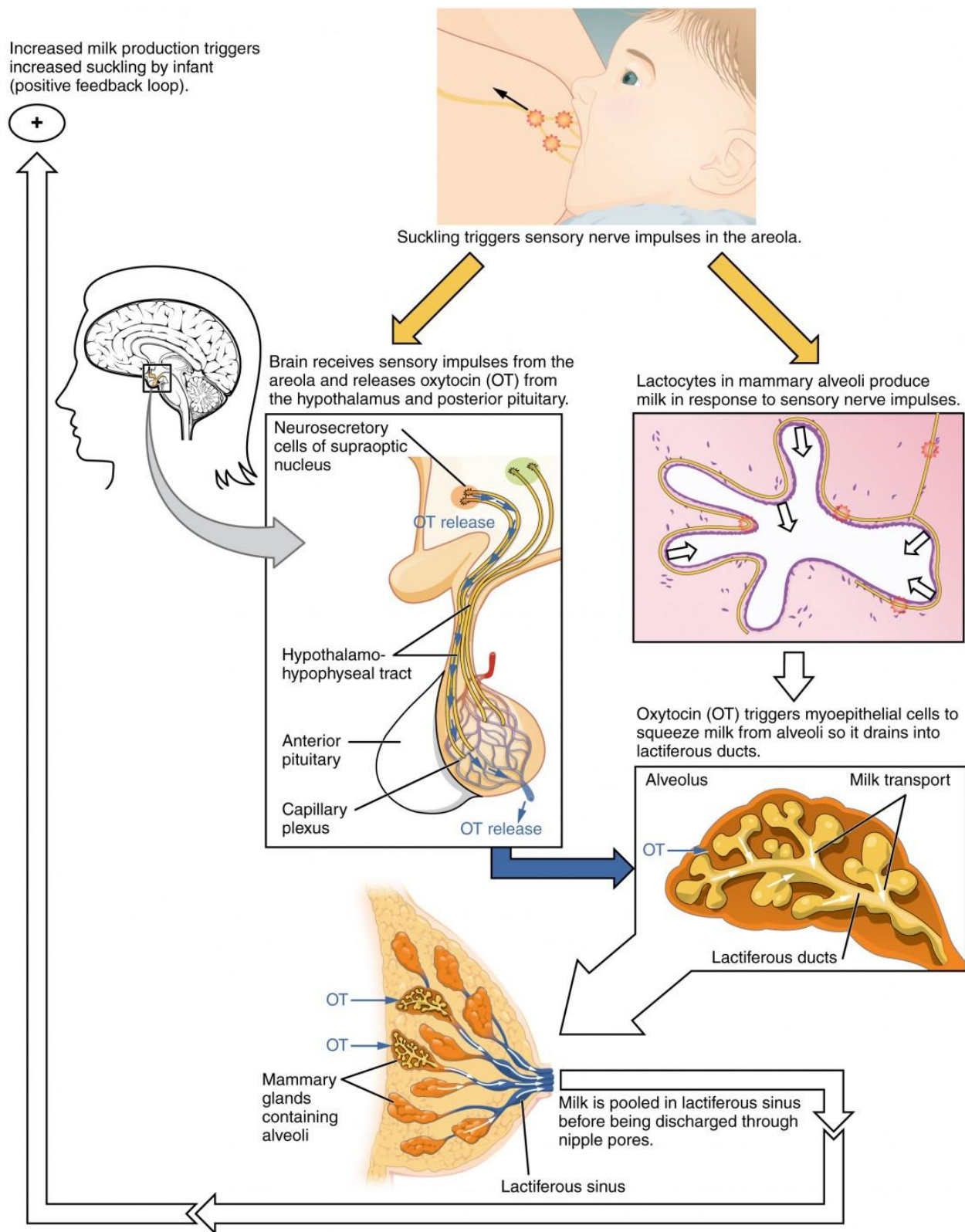


Figure 13: Schematic process of lactation (Anatomy and Physiology).

autocrine feedback mechanisms, rather than being solely dependent on systemic hormonal levels (Cox et al., 1996; Daly et al., 1996; Diaz et al., 1989; Mather et al., 2019; Stevenson et al., 2020). In addition to hormones, autocrine mechanisms that control lactation include alveolar and ductal stretching, as well as the local production of serotonin, insulin-like growth factors (IGFs), and parathyroid hormone-related peptide (Grinman et al., 2022; Krebs et al., 1997).

1.1.10. Mammary Involution Following the Lactation Phase

Involution of the mammary gland is a slow but carefully orchestrated process involving the removal of all milk-secreting epithelial and alveolar cells. This remodeling occurs after the cessation of suckling and the onset of infant weaning, beginning with epithelial cell apoptosis and macrophage-mediated clearance (Monks et al., 2005, 2008). Stromal tissue also disappears together with the regression of lobular dimensions; in the event of a possible future developmental period (such as a subsequent pregnancy), the remodeling of the stroma and gland continues until a state resembling the pre-pregnancy condition is restored (**Figure 14**) (Gallego et al., 2001; Lim et al., 2020; Watson & Khaled, 2008).

The process is divided into several distinct phases. The first phase, also termed the reversible phase, involves the accumulation of milk within the alveolar lumen. Additionally, there is a decrease in lactogenic hormones and an increase in caspase activity, leading mammary epithelial cells to undergo cell death; these dying cells are subsequently eliminated by leucocytes via phagocytosis. Apoptosis in this phase is promoted by the activation of STAT3 and TGF β signaling (Chapman et al., 2000; Humphreys & Hennighausen, 1999). This phase can be reversed if milk-producing cells are reactivated through the resumption of suckling, demonstrating the remarkable plasticity of the mammary epithelium.

The second phase is irreversible. During this stage, caspases and metalloproteinases are activated to remove connective tissues and basement membranes. These enzymes are influenced by molecules of lysosomal origin and by lactogenic hormones. The metalloproteinases—which were inhibited during the first phase by elevated levels of TIMPs (tissue inhibitors of metalloproteinases)—are present in large quantities during this phase, together with serine proteases and plasminogen activator, and proceed to remodel the extracellular matrix and stroma. Following the increase in proteinase activity, epithelial cells detach from the basement membrane,

which is subsequently destroyed (Green & Streuli, 2004). The epithelial cells then dedifferentiate into adipocytes, and the ductal tree is dismantled. This process of ductal regression is not random but is controlled by several molecular factors, including estradiol, progesterone, certain components present in milk, and specific death receptors (Håkansson et al., 1995, 1999).

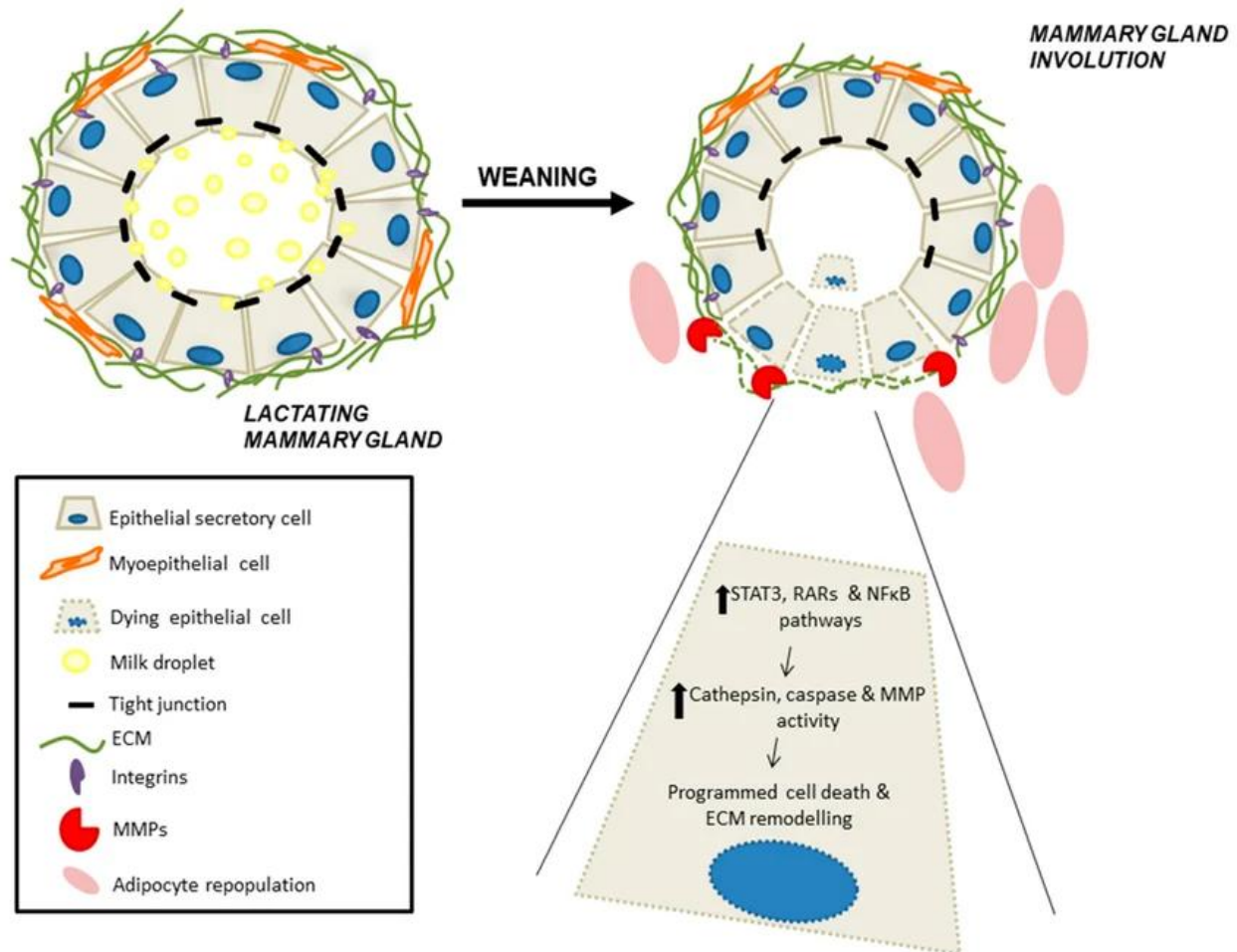


Figure 14: Schematic model of mammary gland involution (M. Teresa Cabezuelo et al., 2019).

The third phase, termed the biosynthetic phase, involves the recruitment of macrophages, phagocytes, and other immune cells for the removal of cellular debris. The stroma is then replenished by adipose tissue cells, restoring the resting state architecture of the gland (**Figure 14**). The entire process of involution thus represents a carefully regulated programme of tissue destruction and regeneration that returns the mammary gland to a quiescent state, primed for potential reactivation during future pregnancies.

1.2. Breast Cancer: Epidemiology, Molecular Subtypes, and the Role of *BRCA1/2*

Breast cancer is the most diagnosed malignancy in women worldwide, with approximately 2.3 million new cases and 685,000 deaths recorded annually (Sung et al., 2021). The disease is characterized by substantial molecular heterogeneity, and gene-expression profiling has delineated at least five intrinsic subtypes — luminal A, luminal B, HER2-enriched, basal-like (triple-negative), and normal-like — each with distinct receptor profiles, clinical behaviors, and therapeutic implications (Perou et al., 2000; Sørlie et al., 2001).

Among the most significant genetic determinants of breast cancer susceptibility are the tumor-suppressor genes *BRCA1* (chromosome 17q21) and *BRCA2* (chromosome 13q12-13), whose germline mutations collectively account for 20–25% of hereditary and 5–10% of all breast cancers (**Figures 15 and 16**) (Miki et al., 1994; Wooster et al., 1995). The *BRCA1* protein is a multifunctional mediator of genomic integrity, participating in DNA double-strand break repair via homologous recombination (HR), cell cycle checkpoint regulation, and chromatin remodeling through its E3 ubiquitin ligase activity with BARD1 (Wang et al., 2000; Hashizume et al., 2001).

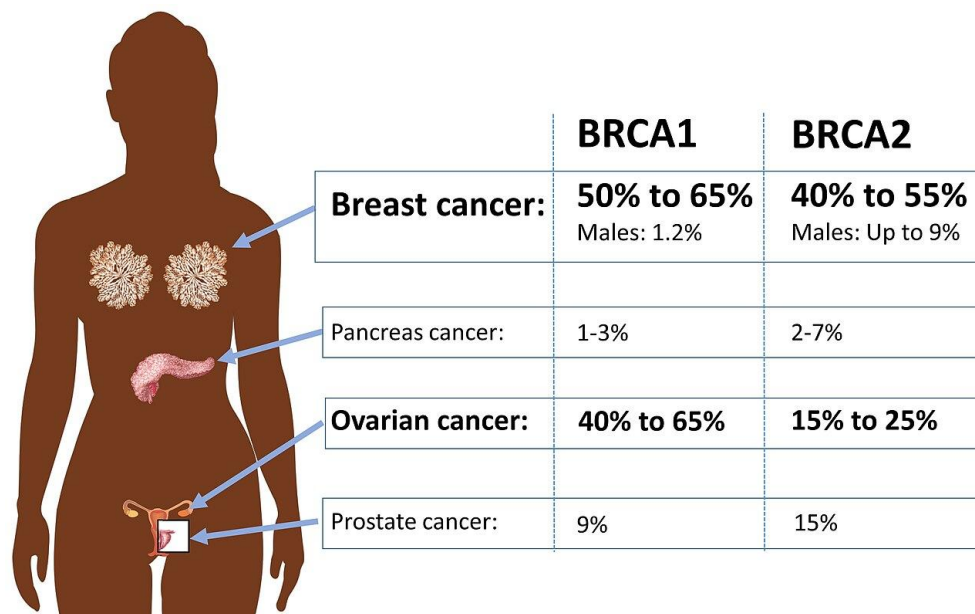


Figure 15: *BRCA1* and *BRCA2* mutations and absolute cancer risk (Image by Mikael Häggström).

Loss of *BRCA1* forces reliance on error-prone repair pathways such as non-homologous end-joining, resulting in chromosomal instability. Carriers of *BRCA1* mutations face a cumulative lifetime breast cancer risk of 60–80%, and their tumors are predominantly basal-like and triple-negative, prompting the hypothesis that *BRCA1* is required for normal luminal progenitor cell differentiation; in its absence, progenitors may stall at an intermediate state that is particularly vulnerable to transformation (Lim et al., 2009).

Critically for hormone receptor biology, *BRCA1* has been shown to directly interact with and inhibit ER α transcriptional activity through its AF-2 domain (Fan et al., 1999, 2001) and to regulate PR signaling in mammary epithelial cells (Ma et al., 2006). These interactions link DNA repair and steroid hormone receptor pathways, suggesting that *BRCA1* loss may simultaneously impair genomic maintenance and dysregulate hormonal signaling. *BRCA2*, by contrast, functions principally as a mediator of RAD51-dependent HR (Pellegrini et al., 2002). *BRCA2*-associated tumors differ phenotypically from *BRCA1*-associated cancers, tending toward the luminal, hormone receptor-positive subtype and resembling sporadic breast cancers morphologically (Wooster et al., 1995). This divergence underscores the distinct roles of the two genes in mammary differentiation and transformation.

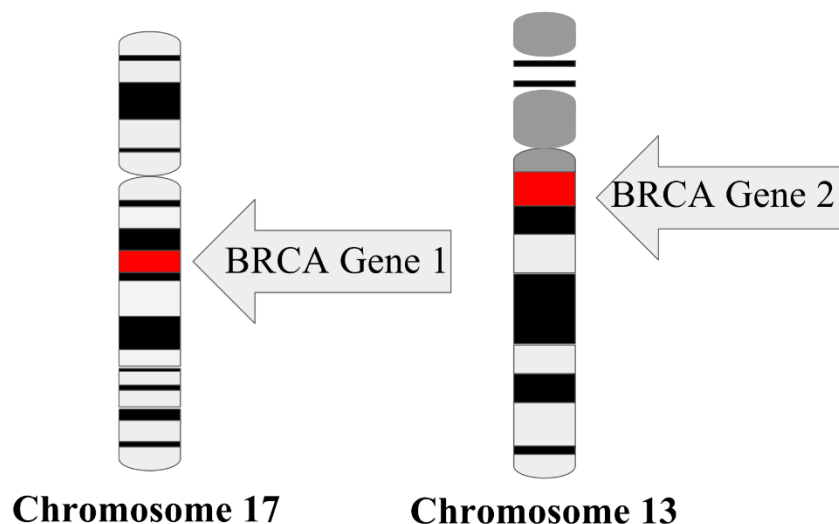


Figure 16: *BRCA1/2* locations (story med).

Despite these advances, the precise mechanisms by which *BRCA1/2* mutations drive tumorigenesis within the context of normal mammary development remain incompletely understood, particularly the interplay between *BRCA1/2* loss, steroid hormone receptor signaling, and mammary progenitor differentiation trajectories. Addressing these gaps necessitates faithful *in vitro* models capable of recapitulating human mammary development and hormonal responsiveness.

1.3. From Two-Dimensional Cultures to Mammospheres and Organoids in Three-Dimensional Cultures to Produce an *in vitro* Mammary Tissue Model

The structural complexity of the mammary gland — with its distinct epithelial cell types, branched architecture and hormone-driven remodeling — has long made the tissue difficult to reconstruct *in vitro*. Early efforts relied on two-dimensional (2D) monolayer cultures derived from patient biopsies, which were attractive for their simplicity, cost and reproducibility and long remained the standard platform for studying cellular function, disease pathogenesis and drug efficacy. Cells of human, animal or synthetic origin can be maintained in plates or flasks, either adherent or in suspension, with defined media providing amino acids, vitamins, growth factors, salts and macromolecules (Setia & Muotri, 2019). However, growth as a flat monolayer on a rigid substrate strips away the three-dimensional organization of the tissue. It also disrupts cell–cell and cell–ECM interactions as well as physiological gradients of nutrients, oxygen, and signaling molecules that characterize the mammary gland *in vivo*.

These constraints drove the development of three-dimensional (3D) culture, in which tissue explants, spheroids and organoids can be supported either by natural scaffolds (collagen, Matrigel, hyaluronic acid), by semi-synthetic or synthetic matrices, or in scaffold-free suspension where the ECM is deposited endogenously by the cells themselves. The scaffold recreates the cell–ECM interactions essential for tissue organization and signaling, while providing a mechanical and biochemical environment that more closely resembles physiological conditions (**Figure 17**).

Among the 3D platforms, spheroids are simple cellular aggregates formed by cell–cell adhesion from established lines or primary cells; they lack a true organized architecture and do not replicate the complex functional features of the tissue of origin, yet they remain widely used in drug screening and in studies of the tumor microenvironment. Organoids, by contrast, are defined as

clusters of cells that self-organize and differentiate into multiple cell types, reproducing the structure and function of the organ of origin. They can be generated from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or adult stem cells through differentiation protocols that reproduce the physiological signals guiding gastrulation and organogenesis *in vivo* (Di Maggio, 2024).

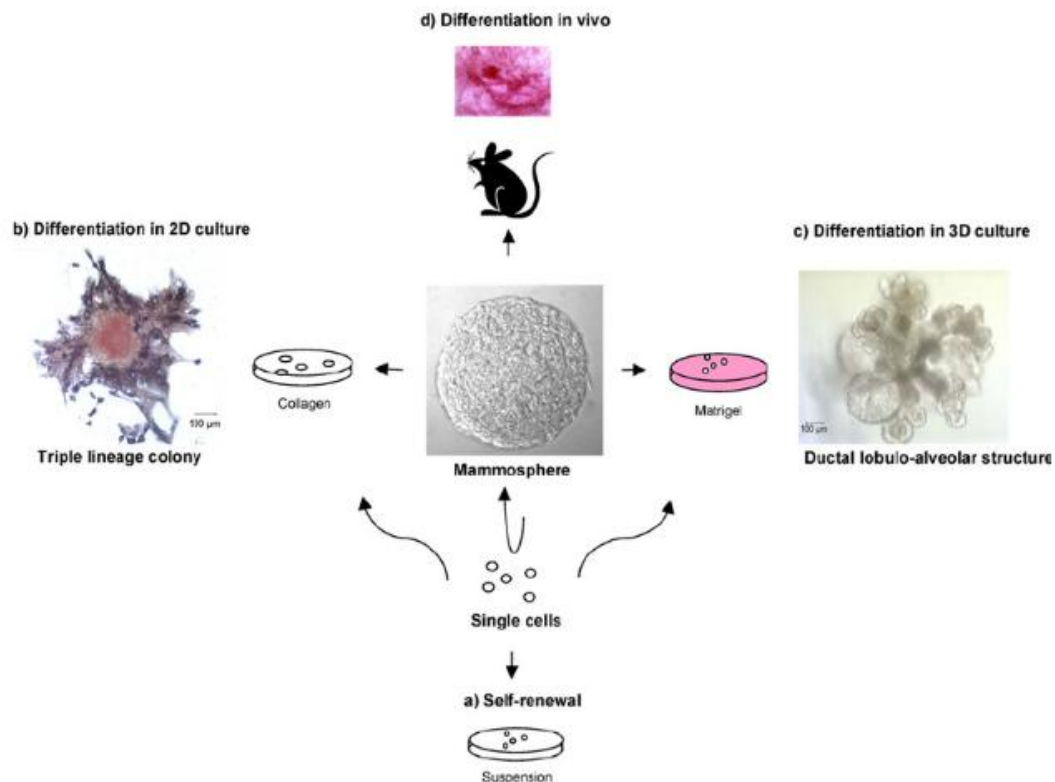


Figure 17: Structure of mammospheres *in vitro* and *in vivo* (Dontu G. et al., 2005).

Within breast research, the first major 3D platform was the mammosphere: spherical colonies formed by mammary cells cultured in suspension under conditions favoring aggregation. Mammospheres proved useful for identifying mammary stem cells within the gland, for propagating ER-positive lineage cells that do not expand in standard mammary-specific media, and for assessing tumor aggressiveness during disease progression (Stingl et al., 2001; Fridriksdottir et al., 2015; Dontu et al., 2003).

Mammospheres harbor a subpopulation of breast cancer stem cells (BCSCs) capable of differentiating along the mammary epithelial lineage. When transplanted into SCID mice, they regenerate tumors, establishing the model as a reference for studying tumor recurrence and progression (Al-Hajj et al., 2004).

Despite this evidence, mammospheres present clear limitations: they are costly and difficult to standardize from primary material, and they display marked cellular, genetic and epigenetic heterogeneity. Comparative analyses performed on luminal (MCF7) and basal (MDA-MB-231) breast cancer lines and on primary tumors from 39 patients revealed major differences in sphere morphology and tumorigenic potential upon xenotransplantation, confirming that mammospheres do not faithfully preserve the architectural and cellular features of the tissue of origin (Clarke et al., 2006). These constraints motivated the transition to organoid-based approaches, which retain stem-cell differentiation potential, reconstruct tissue architecture more faithfully and support long-term propagation with preservation of a normal karyotype (Drost & Clevers, 2017; Sachs et al., 2018).

1.4. Induced Pluripotent Stem Cells (iPSCs)

Alongside conventional cell lines and transgenic animal models, pluripotent stem cells (iPSCs) technology has emerged as a transformative platform for disease modelling and three-dimensional organoid generation.

iPSCs are reprogrammed somatic cells endowed with pluripotency, that is, the capacity to give rise to derivatives of all three germ layers — ectoderm, mesoderm and endoderm. The landmark study of Takahashi and Yamanaka (2007); showed that differentiated cells can be returned to a pluripotent state by the forced expression of four transcription factors — OCT4, SOX2, KLF4 and c-MYC, collectively known as the Yamanaka factors (**Figure 18**). Mechanistically, c-MYC first activates proliferative and metabolic programs while repressing developmental genes; OCT4 and SOX2 subsequently drive the maintenance of pluripotency together with NANOG activation, while KLF4 sustains self-renewal (Buganim et al., 2013).

Early reprogramming protocols relied on integrative retro- and lentiviral vectors, which carry the risk of incomplete transgene silencing, insertional mutagenesis and compromised differentiation

(Okita et al., 2007). Non-integrating alternatives have since become the standard: Sendai virus, which replicates in the cytoplasm without genomic integration (Fusaki et al., 2009), and episomal vectors that yield transgene- and virus-free iPSCs (Thyagarajan et al., 2009), together with mini-circle DNA, PiggyBac transposons, synthetic mRNAs and cell-penetrating recombinant proteins.

iPSCs closely resemble ESCs in stemness-marker expression, colony morphology, telomerase activity and self-renewal, while avoiding the ethical issues linked to human embryo use (Yu et al., 2007). Pluripotency is assessed with the gold-standard teratoma assay and *in vitro* approaches such as immunophenotyping for OCT3/4, SOX2, NANOG, surface antigens and alkaline phosphatase, embryoid body formation, PluriTest bioinformatic profiling and qPCR-based ScoreCard analysis.

Skin fibroblasts and peripheral blood cells remain the most used somatic sources. For non-mobilized blood, specific cytokine cocktails preserve CD34⁺ hematopoietic stem/progenitor cells (HSPCs), which display superior genomic stability and lack the V(D)J rearrangements of committed lymphocytes; however, CD34⁺ cells account for less than 0.01% of adult peripheral blood unless donors receive mobilizing agents such as G-CSF. Among the most promising applications, iPSC-derived organoids have been generated for the intestine (Spence et al., 2011), liver (Takebe et al., 2013), brain (Lancaster et al., 2013), kidney (Low et al., 2019) and mammary gland (Qu et al., 2017), establishing a broad platform for disease modelling, drug screening and regenerative medicine.

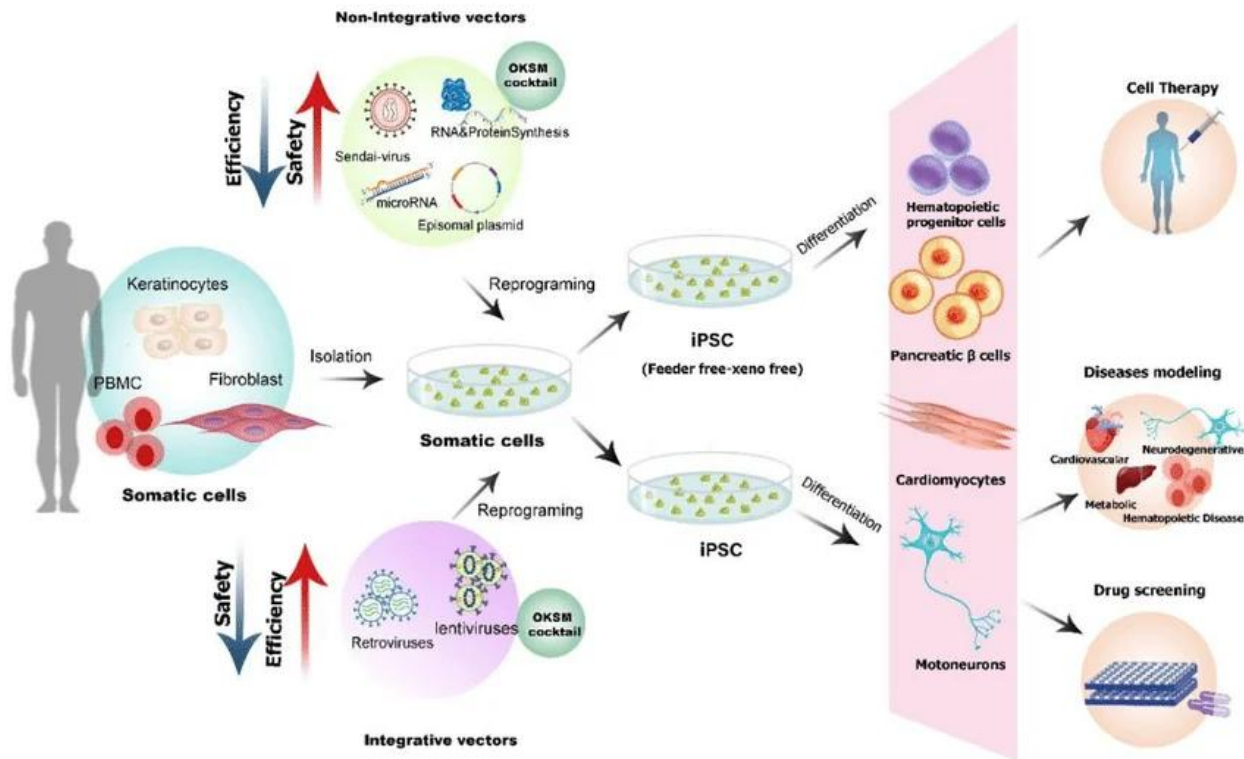


Figure 18: iPSCs reprogramming methods (Elaheh Izady, 2022).

1.4.1. Generation of Mammary Embryoid Bodies

Embryoid bodies (EBs) were first described in the 1950s, when germ-cell tumor cells were observed to assemble into organized aggregates resembling early embryos. With the subsequent derivation of ESCs from the mouse blastocyst, it became clear that these cells could not only generate every somatic and germline lineage but also self-organize into three-dimensional structures reminiscent of early embryonic stages (Martin & Evans, 1975; Pierce et al., 1960). Since then, EBs have been widely exploited to evaluate pluripotency and to dissect lineage specification, which is otherwise difficult to observe *in vivo*. Depending on the stimuli provided, EBs differentiate into derivatives of the three germ layers, for example neurons (ectoderm) or cardiomyocytes (mesoderm), and their formation through hanging-drop, free-suspension or micro-well culture allows tight control of size and morphology.

To investigate mammary development more specifically, so-called mammary embryoid bodies (mEBs) have been introduced with the aim of recapitulating the early steps of mammary

specification *in vitro* (Mikkola, 2007; Propper et al., 2013). The main obstacle is intrinsic to the EB system itself: classical protocols bias cells towards neural ectoderm, whereas mammary development requires progenitors derived from non-neural ectoderm (Schwartz et al., 2008; Zhang et al., 2013). Qu et al. (2017) subsequently demonstrated that iPSCs can be steered along this non-neural route to yield mammary organoids. In practice, the bias is overcome by replacing the conventional EB medium with MammoCult™, a formulation designed to enrich mammary stem cells and epithelial progenitors (Hassiotou et al., 2012), thereby selecting the progenitor population from which the gland arises during embryogenesis (**Figure 19**).

Following the protocol of Qu et al. (2017), iPSCs are detached to obtain a single-cell suspension and seeded into AggreWell™ microwell plates to generate mEBs of 2×10^3 cells. One day after seeding, mEBs are transferred to ultra-low-adherent plates for floating culture in complete MammoCult medium supplemented with heparin and hydrocortisone, allowing the controlled formation of uniform bodies that can subsequently be channeled towards mammary differentiation.

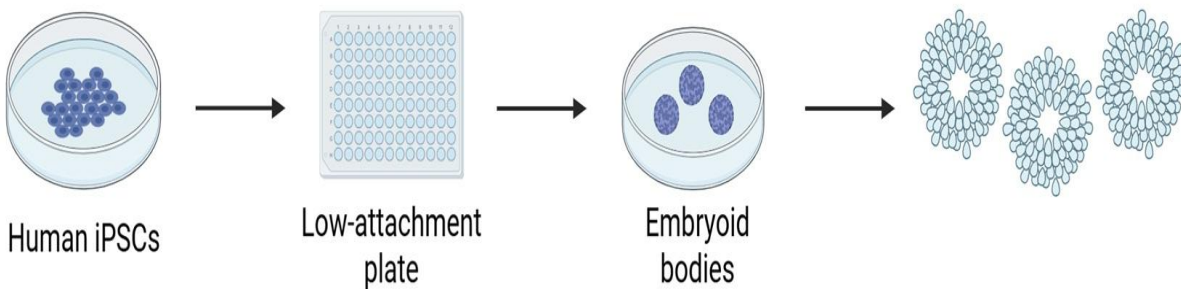


Figure 19: Generation of Mammary Embryoid Bodies (Teresa L-González, 2025).

1.4.2. Generation of Mammary Organoids

Once mEBs have formed, they can be guided into mammary organoids within a three-dimensional scaffold of Matrigel and collagen I. The need for such models emerged when it became clear that murine systems cannot faithfully reproduce the architecture and the molecular wiring of the human mammary gland (J. Lee et al., 2023). Three-dimensional culture has since allowed the direct observation of alveolar assembly, the analysis of signal transduction and gene-expression

regulation, and the dissection of the remodeling events that accompany mammary epithelial cell (MEC) differentiation (Alcaraz et al., 2008; Barcellos-Hoff et al., 1989; Xu et al., 2009).

Two derivation strategies coexist, and the choice between them shapes what the resulting organoid can and cannot model. iPSC-derived organoids, obtained from reprogrammed somatic cells, extend the approach to large and heterogeneous patient populations (Y. Zhang et al., 2013); their main limitation is the absence of a stromal compartment, which constrains the reproduction of cell–microenvironment interactions and has prompted ongoing efforts to reintroduce stromal cells into these systems (J. Lee et al., 2023). Patient-tissue-derived organoids, in turn, preserve the full cellular heterogeneity of the source. Rosenbluth et al. (2020) demonstrated by flow cytometry that these cultures retain luminal, basal and stem populations, and display ductal and alveolar morphologies that closely mirror native tissue; crucially, they also preserve the stromal cells that support epithelial stemness and reconstruct the local microenvironment.

Beyond what mammospheres could offer, mammary organoids enable pharmacological screening, support the generation of patient-derived biobanks, and provide a tractable framework for dissecting the morphological and molecular changes that remain poorly characterized during lactation and involution (Sachs et al., 2018).

1.4.3. Embedding in Matrigel

A critical step in mammary organoid generation is their inclusion within a three-dimensional gel whose composition approximates that of the mammary extracellular matrix (ECM) *in vivo* (Shamir & Ewald, 2014; Simian et al., 2001). Organoids are embedded in the matrix and subsequently overlaid with culture medium, so that the gel simultaneously provides mechanical support and the biochemical cues that characterize the physiological ECM (Hynes & Naba, 2012).

Collagen I was already in use during the 1970s for mammary epithelial cell (MEC) culture, and the adoption of a physiologically relevant three-dimensional matrix proved essential for functional differentiation, underscoring the significant role of cell–matrix interactions in the development of the gland (Emerman et al., 1977). Later work showed that MEC proliferation and differentiation also depend on the conformation of the collagen matrix, whether anchored to a substrate or suspended in dome-like structures (Aggeler et al., 1991). Contemporary protocols are largely based on commercial Matrigel and collagen I mixtures, which supply basement-membrane proteins such

as laminin and collagen IV and allow organoids to self-assemble into clusters of stratified mammary epithelium. The addition of defined growth factors stimulates branching morphogenesis, a key step in mammary gland maturation (Jamieson et al., 2017) (**Figure 20**). Matrix stiffness and the ratio between Matrigel and collagen I further shape organoid morphology and growth (Mohan et al., 2021; Nguyen-Ngoc & Ewald, 2013; Paszek et al., 2005).

Several embedding techniques have been developed over the years, each tailored to a specific aspect of mammary biology. In dome culture, a small hemispherical Matrigel drop containing the cells is deposited at the center of the culture dish; the hemispherical geometry itself favors controlled three-dimensional growth. In droplet culture, cells are suspended in microdroplets of Matrigel or collagen that solidify into independent microenvironments, keeping each organoid isolated from the others. The hanging-drop method exploits gravity: the cell-containing drops are placed inverted on the lid of the plate so that aggregation is favored and spheroids form before the cells are embedded in the matrix. Finally, inverted organoids reproduce the apico-basolateral polarity of the native epithelium more faithfully, with the basal side-oriented outwards and the apical side enclosed within the structure (Mertz et al., 2023; Kim et al., 2020; Foty, 2011). Together, these approaches yield models that more closely recapitulate the tissue transitions of pregnancy, lactation, and involution (Feigman et al., 2020; Sumbal et al., 2020).

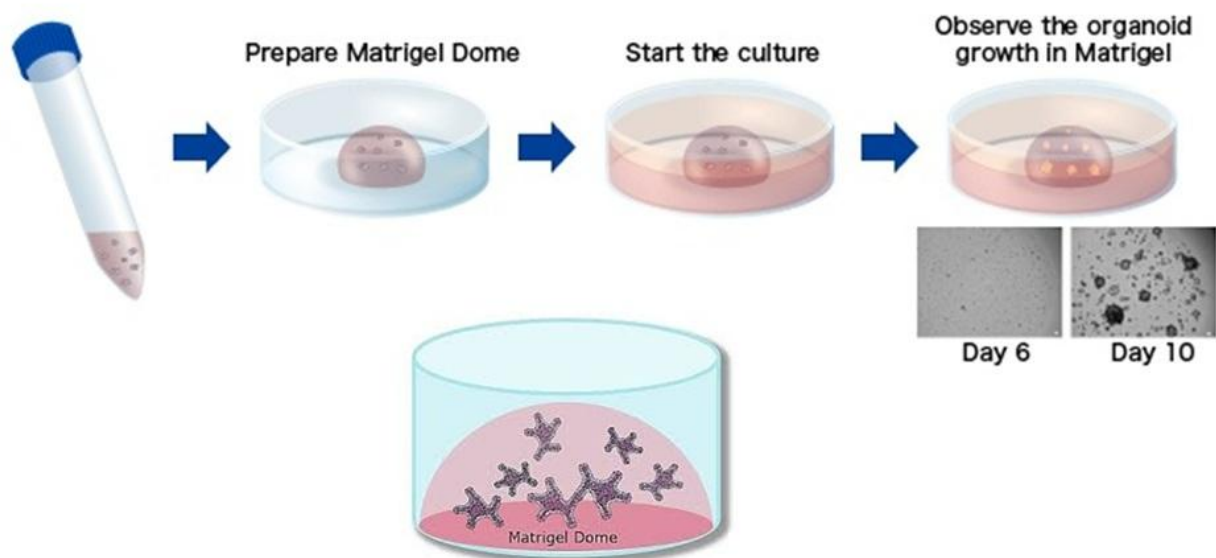


Figure 20: Embedding EBs in Matrigel Dome (MBL).

The contribution of the stromal compartment has been further established through co-culture experiments, for instance by combining mammary organoids with fibroblasts to enrich the matrix and improve the microenvironment (Koledova & Lu, 2017; Krause et al., 2008). In parallel, 3D printing has been introduced to increase reproducibility and standardization, allowing the precise and controlled positioning of cells within hydrogels (Reid et al., 2018).

1.5. Steroid Hormone Receptors in Stem Cells Biology: From Embryonic Stem Cells to Induced Pluripotent Stem Cells

Steroid hormones, particularly estrogen and progesterone, regulate a broad spectrum of physiological processes, including cell differentiation and development, cellular homeostasis, and reproduction (Baker, 2003; Molenda et al., 2003). Estrogens are primarily synthesized from androgen precursors in ovarian granulosa cells and the placenta in females, in the testis in males, and in non-glandular tissues such as fat and bone in both sexes (Vermeulen et al., 2002). Progesterone, in turn, is produced by ovarian granulosa cells, the adrenal glands, and the corpus luteum during the menstrual cycle, by the placenta in females, and by the adrenal glands in both males and females (Guennoun et al., 2015). These hormones exert their biological effects by binding to intracellular receptors — ER α/β and PR — which belong to the nuclear receptor superfamily of ligand-activated transcription factors (Taubenheim et al., 2021). Both estrogen and progesterone fulfil distinct roles during embryonic development (Pepe & Albrecht, 2009) and, postnatally, contribute to the maturation of immune cells, exerting notable effects on the bone marrow and on hematopoietic stem cells and progenitor cells involved in the development and maturation of hematopoietic lineages (Smithson et al., 1998; Guo et al., 2018).

The progesterone receptor exists as two principal isoforms, PR-A and PR-B, whose transcription is governed by distinct estrogen-inducible promoters with alternative AUG initiation codons; consequently, PRs are direct downstream targets of ERs (Anderson, 2002). A noteworthy functional distinction between the two isoforms is that PR-A can act as a dominant repressor of both PR-B and ER in a promoter- and cell-type-specific manner (Vegeto et al., 1993; Wen et al., 1994). Of further significance, the DNA-repair tumor-suppressor protein *BRCA1* has been shown

to interact with and regulate ER α and PR transcriptional activation (Fan et al., 1999, 2001; Ma et al., 2006), thereby linking steroid hormone receptor signaling with the DNA damage response pathways that are characteristically disrupted in hereditary breast cancer (**Figure 21**).

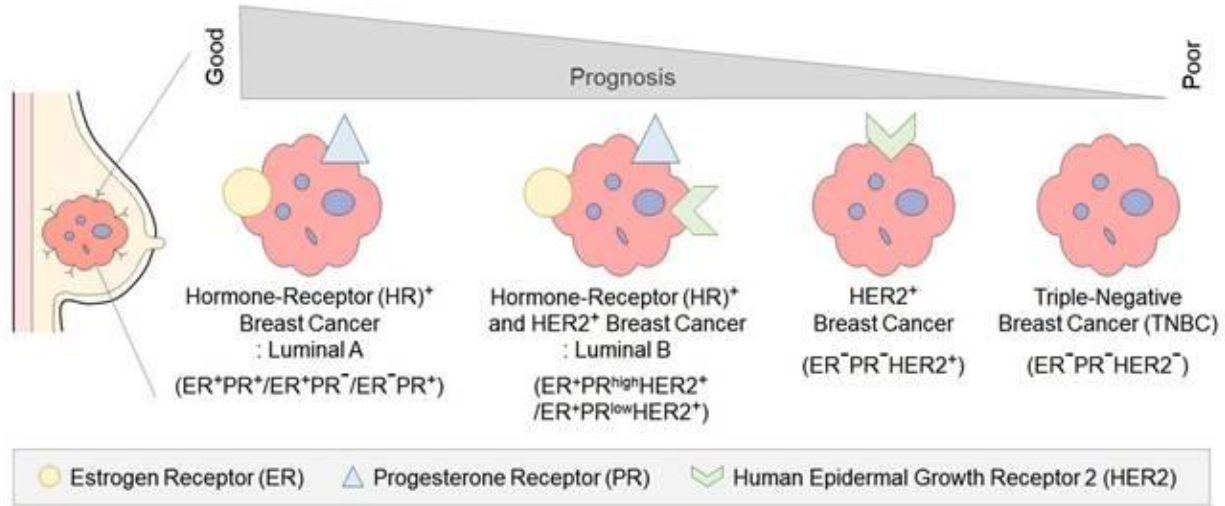


Figure 21: Classification of breast cancer subtypes (Eun-Jin Go, 2025).

Steroid hormone-related proteins, including ER α/β and PR, have been reported to be expressed in embryonic stem cells and during early development in mice through the blastocyst stage (Hong et al., 2004; Hou & Gorski, 1993). In particular, the importance of ER β in self-renewal and pluripotency has been further elucidated in subsequent studies (Loh et al., 2006; Ivanova et al., 2006). Progesterone has been shown to be essential for the differentiation of ESCs during human embryonic development (Gallego et al., 2009), an action mediated by PR-A expressed in ESCs (Mierzejewska et al., 2015; Abdelbaset-Ismail et al., 2016). Moreover, progesterone has been demonstrated to both induce (López-González et al., 2011) and inhibit (Kang et al., 2016) the differentiation of ESCs into specific lineages, as well as to regulate the development and physiology of steroid-hormone-responsive organs (Goletiani et al., 2007; Kaore et al., 2012; Hagan et al., 2012; Scarpin et al., 2009).

Despite this growing body of evidence establishing the expression and functional relevance of steroid hormone receptors in embryonic stem cells, the status of these receptors in induced pluripotent stem cells had remained unexplored prior to the work presented in this thesis. Given that iPSCs are increasingly employed as platforms for disease modelling – including for hormone-responsive tissues such as the mammary gland – understanding whether PR and ER α/β are

expressed in undifferentiated iPSCs and how their expression changes during lineage-specific differentiation represents a critical gap in current knowledge. The relationship between PR and its known downstream target genes, such as the progesterone-inducible gene CK5 (Axlund et al., 2013) and GATA3, whose expression is negatively regulated by PR activation through transcriptional repression (Izzo et al., 2014), further underscores the importance of characterizing PR dynamics in iPSC-based differentiation systems. Addressing this gap formed a central aim of the present study, and the findings are presented in the Results chapter of this thesis.

1.6. iPSC-Derived Mammary Organoids as Tools for Cancer Research and Translational Applications

Faithful *in vitro* models of the mammary gland are essential for understanding breast cancer biology, particularly in the context of hereditary predisposition associated with *BRCA1* and *BRCA2* mutations. Patient-derived mammary organoids can preserve the cellular heterogeneity of the tissue of origin, including luminal, basal, and stem-cell populations, and recapitulate ductal and alveolar structures (Rosenbluth et al., 2020). However, their utility is limited by the difficulty of obtaining tissue from healthy *BRCA* mutation carriers prior to cancer development, the restricted expandability of primary samples, and the challenge of culturing all molecular subtypes with equal efficiency.

iPSC-derived mammary organoids address many of these constraints. iPSCs can be generated from readily accessible somatic cells – peripheral blood or skin fibroblasts – of any individual, including *BRCA1/2* mutation carriers, enabling the creation of patient-specific models that expand indefinitely and differentiate into mammary-like tissues on demand. This makes them particularly suited for studying pre-malignant stages of breast cancer and tumor-initiating events in the context of intact tissue architecture, both of which are essentially inaccessible through conventional biopsies. iPSC technology further permits CRISPR-Cas9-mediated generation of isogenic cell lines differing only in specific mutations, eliminating genetic background variation and enabling precise genotype-phenotype attribution. Given the direct interactions between *BRCA1* and both ER α and PR signaling (Fan et al., 1999, 2001; Ma et al., 2006), such models could illuminate how hormone receptor biology is perturbed at the earliest stages of *BRCA*-associated tumorigenesis (**Figure 22**).

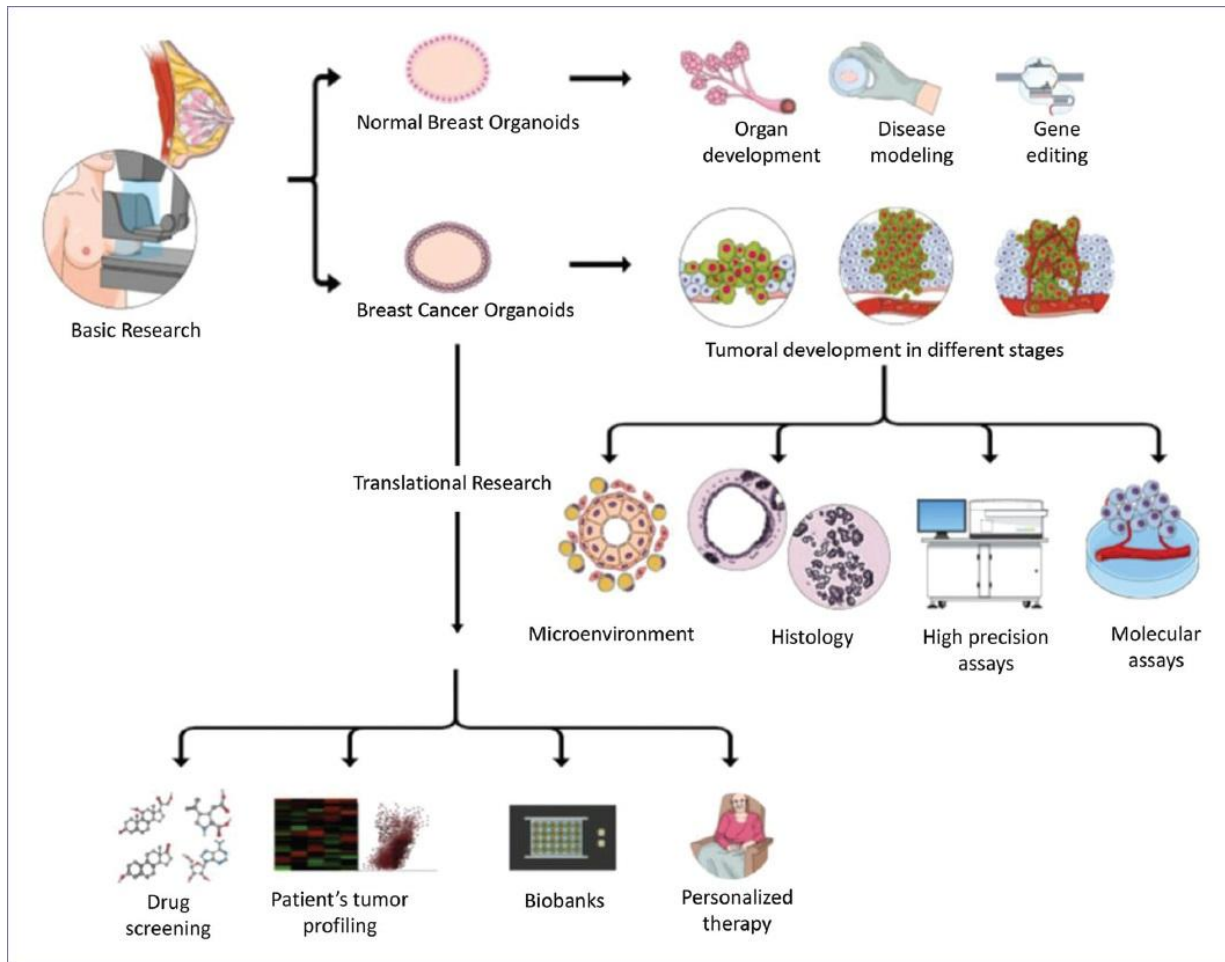


Figure 22: Applications of organoids in basic and translational Breast Cancer (BC) research (Ignacio U. Macías-Paz, 2022).

These organoids also offer a unique platform for investigating the developmental origins of breast cancer susceptibility by recapitulating sequential stages of mammary differentiation from pluripotency through embryoid body formation to branching morphogenesis. Understanding how *BRCA1/2* mutations alter differentiation trajectories, lineage marker expression (CK5, CK7, CK8, CK14, CK18, GATA3, TP63), and steroid hormone responsiveness may reveal novel therapeutic targets for high-risk individuals. From a translational perspective, these models could be employed for pharmacological screening to identify compounds that restore normal differentiation in *BRCA*-deficient cells, for testing chemopreventive agents, for studying the effects of environmental estrogens and endocrine disruptors on mammary development, and for developing personalized therapeutic strategies and risk assessment tools tailored to individual patients' genetic backgrounds and *in vitro* differentiation profiles.

Looking ahead, the optimization of the stromal microenvironment, the reduction of neural ectoderm contamination, and the extension of differentiation protocols to encompass the secretory and lactation phases will represent critical advances for improving model fidelity. The integration of iPSC-derived mammary organoids with microfluidic organ-on-a-chip technologies, single-cell transcriptomics, and spatial proteomics will further enhance their capacity to recapitulate the complexity of human mammary tissue and to serve as platforms for precision medicine in breast cancer research.

CHAPTER 2

Aim of Research

Breast cancer is among the most common malignancies in women, and germline mutations in the tumor-suppressor genes *BRCA1* and *BRCA2* account for the best-characterized hereditary predisposition to the disease. The mammary gland itself is a highly dynamic organ, remodeling across puberty, pregnancy, lactation and involution under hormonal control and through the coordinated activity of stem, progenitor, luminal and basal cell compartments. Addressing both its physiological development and the earliest event of hereditary tumorigenesis calls for *in vitro* systems able to capture this cellular and hormonal complexity — a requirement that two-dimensional cell lines, primary biopsy-derived cultures and murine models only partially fulfill.

In this setting, induced pluripotent stem cells (iPSCs) offer a powerful model system. Their capacity for unlimited self-renewal and multilineage differentiation makes them a versatile source of patient-specific mammary epithelium, which can then be guided into three-dimensional mammary-like organoids reproducing luminal–basal compartmentalization and hormone responsiveness.

Within this framework, the aim of the present PhD project was to establish a reproducible iPSC-based platform for modelling the human mammary gland *in vitro*, through a staged differentiation strategy proceeding from pluripotency to mammary embryoid body (mEBs) formation and, ultimately, to three-dimensional mammary-like organoid maturation. In parallel, the expression of estrogen receptors ER α and ER β and of the progesterone receptor (PR) was investigated both in the undifferentiated iPSCs and throughout the differentiation process, with the purpose of clarifying how steroid hormone receptor expression is regulated during the progressive acquisition of mammary identity. The broader objective of this work was to generate functional mammary-like organoids suitable as disease models of *BRCA1/2*-associated breast cancer and, in the long term, to provide a platform for pharmacological screening, patient-specific disease modelling, and gene-editing applications.

This research was carried out at the Angelo Nocivelli Institute for Molecular Medicine, Department of Molecular and Translational Medicine, University of Brescia, and ASST Spedali Civili of Brescia.

Chapter 3

Materials and Methods

3.1. Cell lines and culture conditions

Four induced pluripotent stem cell (iPSC) lines, derived from two distinct somatic origins and generated through four independent reprogramming strategies, were used in this work (Table 1). The Episomal iPSC line, used throughout the differentiation pipeline and extensively characterized, was purchased from Thermo Fisher Scientific (cat. no. A18945, Waltham, MA, USA); it was reprogrammed from cord blood–derived CD34+ progenitors by episomal-vector delivery of seven factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28 and SV40 T). Three additional iPSC lines, BJ, 253-G1 and F3, were used for the comparative receptor-expression analyses: BJ was reprogrammed from human neonatal foreskin fibroblasts (BJ strain, ATCC) using the CytoTune-iPS 2.0 Sendai Reprogramming Kit; 253-G1 was obtained from human fibroblasts by retroviral transduction; and F3 was generated from human fibroblasts by lentiviral transduction.

All iPSC lines were propagated in adhesion on plates (Costar) pre-coated with Matrigel (Corning Inc., Corning, NY, USA) and maintained with daily medium changes using NutriStem[®] hPSC XF Medium (Sartorius AG, Göttingen, Germany) supplemented with 10 ng/mL basic fibroblast growth factor (bFGF; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Colonies were mechanically passaged by manual colony picking every 4–5 days onto freshly Matrigel-coated plates and maintained at 37 °C under a humidified atmosphere of 5% CO₂. Colony morphology was monitored routinely by phase-contrast microscopy at each medium change.

Two human breast cancer cell lines were used as expression controls for the prolactin receptor (PRLR) and for the steroid hormone receptors. MCF7 (ER α + / ER β - / PR+) served as positive control for PR and PRLR expression; MDA-MB-231 (ER α - / ER β + / PR-) served as negative control for PR and positive control for ER β . Both lines were grown in adhesion in DMEM (code ECB7501L; Euroclone S.p.a., Pero, Italy) — with the exception of MDA-MB-231, which was grown in RPMI-1640 (code R0883; Sigma-Aldrich, USA) — supplemented in each case with 10% fetal bovine serum (FBS; Euroclone S.p.a.), 1% L-glutamine and 1% penicillin/streptomycin

(Euroclone S.p.a.). Parental BJ human foreskin fibroblasts, used as a somatic control for receptor expression, were maintained in the same DMEM-based medium.

iPSC line	Parental cell	Reprogramming strategy
Episomal (cat. no. A18945)	Cord blood-derived CD34+ progenitors	Episomal vector (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, SV40 T)
BJ	Human neonatal foreskin fibroblasts (BJ strain, ATCC)	CytoTune-iPS 2.0 Sendai Reprogramming Kit
253-G1	Human fibroblasts	Retroviral transduction
F3	Human fibroblasts	Lentiviral transduction

Table 1. iPSC cell lines used in this thesis work, with their respective parental cells and reprogramming strategies.

3.2. Mycoplasma detection

Because the iPSC lines were maintained in continuous culture over extended periods, the absence of mycoplasma contamination was verified periodically by end-point PCR on medium sampled from wells at high confluence. Amplification products were resolved by electrophoresis on an agarose gel. The composition of the amplification mix is given in Table 2.

Reagent	Volume
Buffer 10×	2.5 μ L
MgCl ₂ (25 mM)	2 μ L
Betaine	2.5 μ L
dNTPs (10 mM)	1 μ L
Forward primer (10 μ M)	1 μ L

Reagent	Volume
Reverse primer (10 μ M)	1 μ L
AmpliTaq Gold (5 U/mL)	0.5 μ L
H ₂ O	11.5 μ L
Sample / blank	3 μ L
Final volume	25 μL

Table 2. Composition of the PCR amplification mix for mycoplasma detection.

3.3. Generation of mammary embryoid bodies (mEBs) from iPSCs

Once iPSC colonies reached confluence, they were non-enzymatically detached and dissociated with Gentle Cell Dissociation Reagent (StemCell Technologies Inc., CA), counted, and seeded onto AggreWell™ Microwell Plates (StemCell Technologies Inc., CA). Plates had been pre-treated with an anti-adherence rinsing agent to prevent cell attachment to the plate surface; each well contains 300 microwells. A total of 1.2×10^6 cells were seeded per well to generate approximately 300 spheroids of around 4,000 cells each. Cells were centrifuged into the microwells at 1,800 rpm for 5 min, which ensured uniform aggregate formation. After 24 h, only single, non-fused spheroids of comparable size and morphology were manually selected under phase-contrast microscopy and transferred to 6-well ultra-low adhesion plates (Corning Inc., USA) for floating culture.

Floating mEBs were maintained for 10 days in complete MammoCult™ medium (StemCell Technologies), a formulation specifically designed for mammosphere culture that biases non-neural ectodermal specification while suppressing the neural lineage. Full medium replacement was performed every 48-h. medium composition is summarized in Table 3. On day 10, representative of peak non-neural ectodermal enrichment, mEBs were harvested either for molecular analyses or for subsequent three-dimensional matrix embedding (section 3.4).

Reagent	Final concentration	Volume (for 50 mL)
MammoCult Basal Medium	—	45 mL
MammoCult Proliferation Supplement	—	5 mL
Heparin (2 ng/ μ L)	4 μ g/mL	100 μ L
Hydrocortisone (96 ng/ μ L)	0.48 μ g/mL	250 μ L

Table 3. Composition of the complete MammoCult™ medium used during the floating mEBs phase (days 0–10).

3.4. Three-dimensional mammary-like organoid culture

On day 10 of differentiation, mEBs were embedded in a three-dimensional matrix composed of Matrigel (Corning) and type I collagen (Sigma-Aldrich, USA) to generate dome cultures, following the protocol of Manganelli et al. (2024) with minor adjustments. A mix of all matrix components except Matrigel was prepared on ice; the pH was verified with litmus paper and adjusted to neutral with 1 M NaOH ($0.023 \times$ initial volume of type I collagen), after which Matrigel was added under neutral conditions. The composition of the embedding matrix is reported in Table 4. After 30 min on ice, each spheroid was collected with the embedding solution and plated as an individual dome on cell-culture plates. Domes were allowed to solidify for 30 min at 37 °C before medium addition.

Reagent	Final concentration	Volume
Matrigel (11.4 mg/mL)	4.5 mg/mL	19.7 μ L
Type I collagen (3.43 mg/mL)	1.5 mg/mL	21.8 μ L
PBS 10×	1×	5 μ L

Reagent	Final concentration	Volume
NaOH (1 M)	—	$0.023 \times$ initial volume of type I collagen
H ₂ O	—	3 μ L
Total volume		50 μL

Table 4. Composition of the Matrigel/type I collagen matrix used for three-dimensional embedding of day 10 mEBs.

The three-dimensional culture was then maintained in two sequential differentiation phases. During the mammary-commitment phase (days 10–15), domes were cultured in complete EpiCult-B medium (code 05602; StemCell Technologies) supplemented with parathyroid hormone–related protein (PTHrP, 100 ng/mL; Sigma-Aldrich, USA) to expand both bipotent and lineage-restricted luminal and myoepithelial progenitors (Table 5). From day 15 onward, the medium was replaced with the branchio-alveolar differentiation medium (Table 6), containing hydrocortisone, insulin, FGF10 and HGF, to promote branching morphogenesis and alveolar maturation. This medium was refreshed every 3 days until day 35, the time point at which organoids were collected for histological, immunohistochemical and molecular characterization. Organoid morphology was documented throughout by phase-contrast microscopy.

Reagent	Final concentration	Volume (for 100 mL)
EpiCult-B Basal Medium	—	100 mL
EpiCult-B Proliferation Supplement	—	1 mL
L-glutamine (200 mM)	2 mM	1 mL
PTHrP (100 ng/mL)	100 ng/mL	100 μ L

Table 5. Composition of the Mammary Committed Medium (days 10–15).

Reagent	Final concentration	Volume (for 50 mL)
EpiCult-B complete medium	—	50 mL
Hydrocortisone (96 mg/ μ L stock)	1 μ g/mL	500 μ L
Insulin (20 mg/mL)	10 μ g/mL	25 μ L
FGF10 (100 ng/ μ L; Peprotech)	50 ng/mL	25 μ L
HGF (100 ng/ μ L; Peprotech)	50 ng/mL	25 μ L

Table 6. Composition of the Branchio/Alveolar Differentiation Medium (days 15–35).

3.5. RNA extraction and reverse transcription

Samples were collected at defined time points throughout the differentiation trajectory, namely day 0 (undifferentiated iPSCs), days 2, 5 and 10 (mEBs) and day 20 and day 35 (mammary-like organoids). Total RNA from iPSCs and mEBs was extracted with the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. On-column DNase digestion with TURBO™ DNase (Invitrogen; Thermo Fisher Scientific, Inc.) was applied to remove residual genomic DNA.

Mature organoids required pre-treatment before RNA extraction. Individual organoids were released from the Matrigel/collagen dome by digestion with Collagenase I (1 $\times$) at 37 °C, and RNA was subsequently isolated from the single-organoid lysate using TRIzol™ reagent (code 15596026; Invitrogen™). RNA concentration and purity were assessed on a TECAN® Infinite M200 spectrophotometer (or equivalent Tecan platform; Tecan Group Ltd., Männedorf, Switzerland), recording absorbance at 260 and 280 nm. A 260/280 ratio above 2.0 was considered acceptable; lower values indicative of protein or phenol contamination led to sample rejection.

For each sample, 500 ng (thesis protocol) or 1 μ g (multi-line comparative experiments) of total RNA was reverse-transcribed to cDNA using the ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI, USA) following the manufacturer's protocol. Random

primers were first annealed to the RNA template at 70 °C for 5 min and cooled on ice for a further 5 min (Table 7); the reverse-transcription reaction was then assembled as in Table 8 and cycled on a thermocycler as follows: 25 °C for 5 min (primer annealing), 42 °C for 60 min (cDNA synthesis), 70 °C for 15 min (enzyme inactivation), and 4 °C hold. cDNA was stored at –20 °C until use.

Reagent	Volume
RNA sample	X μ L (adjusted on the basis of the final cDNA amount required and the RNA concentration)
Random primers (500 μ g/mL)	1 μ L
Nuclease-free water	X μ L to final volume
Final volume	10 μL

Table 7. Components of the first reverse-transcription mix (RNA–primer annealing step).

Reagent	Volume
Buffer 5×	4 μ L
MgCl ₂ (25 mM)	2.4 μ L
dNTPs mix (10 mM)	1 μ L
Recombinant RNasin Ribonuclease Inhibitor (40 U/ μ L)	0.5 μ L
ImProm-II™ Reverse Transcriptase	1 μ L
Nuclease-free water	1.1 μ L
RNA + random primers (first mix)	10 μ L
Final volume	20 μL

Table 8. Components of the second reverse-transcription mix (cDNA synthesis step).

3.6. End-point RT-PCR for pluripotency and terminal-differentiation markers

End-point RT-PCR was used for two purposes. First, at the quality-control stage, the stemness of the Episomal iPSC line at successive passages was verified by amplifying the Yamanaka factors OCT4, SOX2, KLF4 and c-MYC together with NANOG. Second, at the end of the differentiation pipeline, expression of the prolactin receptor (PRLR) and progesterone receptor (PR) transcripts was assessed in undifferentiated iPSCs, day 10 mEBs and in mature organoids generated from two representative iPSC lines (Episomal and BJ); MCF7 and MDA-MB-231 cells served as positive and negative controls, respectively, and GAPDH was amplified in parallel as the internal reference.

The reaction mix was assembled as in Table 9, and amplification products were resolved on agarose gel. A no-template water blank was included in every run to exclude contamination.

Reagent	Volume
Buffer 10×	2.5 μ L
MgCl ₂ (25 mM)	2 μ L
dNTPs (10 mM)	1 μ L
Forward primer (10 μ M)	1 μ L
Reverse primer (10 μ M)	1 μ L
AmpliTaq Gold (5 U/mL)	0.5 μ L
cDNA / blank (5 ng/ μ L)	4 μ L
H ₂ O	13 μ L
Final volume	25 μL

Table 9. Composition of the end-point PCR reaction mix (per gene).

3.7. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was the primary analytical approach for transcriptional analyses presented in this thesis. It was applied to (i) monitor the molecular transition from pluripotency toward a mammary-competent progenitor during mEBs differentiation, analyzing iPSC and mEBs samples collected at days 0, 2, 5 and 10; (ii) follow the temporal modulation of the prolactin receptor (PRLR) in dome cultures at day 5 and day 20; (iii) compare estrogen receptor α , estrogen receptor β and progesterone receptor transcript levels across the four iPSC lines relative to MCF7 and MDA-MB-231 controls; and (iv) quantify luminal, basal, hormone-receptor and pluripotency markers longitudinally across the three key differentiation stages (undifferentiated iPSCs, day 10 mEBs and day 20 mammary-like organoids).

Reactions were performed using the iTaq Universal SYBR[®] Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the reagent composition shown in Table 10. After sample loading, plates were sealed with an adhesive film, centrifuged at 1,800 rpm for 2 min, and run on a CFX96[™] Touch Real-Time PCR Detection System (Bio-Rad) under the following cycling programme: 98 °C for 30 s (initial denaturation); 39 cycles of 95 °C for 5 s (denaturation) and 60 °C for 30 s (combined annealing and extension); followed by a melting-curve ramp from 65 °C to 95 °C at 0.5 °C per 5 s to confirm amplicon specificity. Data was analyzed with Bio-Rad CFX Manager (v.3.1) software.

Relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method. For the time-course experiments carried out within the thesis differentiation pipeline (mEBs commitment analysis, PRLR expression kinetics during dome culture, end-point terminal-differentiation analysis), the housekeeping gene GAPDH was used as normalizer and the day 0 iPSC sample as calibrator. For the multi-iPSC-line comparative experiments and the longitudinal receptor/marker analyses performed in parallel with the published Manganelli et al. (2024) study, β -actin (Hs.PT.56a.19461448.g; IDT, Inc., Coralville, IA, USA) was used as the reference transcript, with MCF7 or undifferentiated iPSCs used as calibrator as appropriate to the experimental design. Each experiment was run in technical triplicates and repeated in at least three independent biological replicates.

Reagent	Volume
iTaq Universal SYBR® Green Supermix (2×; Bio-Rad)	12.5 µL
Forward primer (5 µM)	1 µL
Reverse primer (5 µM)	1 µL
H ₂ O	8.5 µL
cDNA sample (5 ng/µL)	2 µL
Final volume	25 µL

Table 10. Composition of the qRT-PCR reaction mix.

The complete primer panel used across the qRT-PCR and end-point RT-PCR experiments is given in Table 11. Primer pairs targeting the steroid hormone receptors (ER α , ER β , PR) and the luminal and basal cytokeratin's were those validated in Manganelli et al. (2024); the remaining primers for pluripotency, non-neural ectodermal, neural-ectodermal and mammary-commitment markers were designed and validated in-house.

Gene	Forward (5'→3')	Reverse (5'→3')	Functional category
GAPDH	GTCAACGGATTGTCGTATT	CATGGGTGGAATCATATTGGAA	Reference
β-actin (Hs.PT.56a.19461448.g)	Proprietary assay (IDT)	Proprietary assay (IDT)	Reference
OCT4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT	Pluripotency
SOX2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCTCGTTTT	Pluripotency
KLF4	GATGAACTGACCAGGCACTA	GTGGGTCATATCCACTGTCT	Pluripotency
c-MYC	TGCCTCAAATTGGACTTTGG	GATTGAAATTCTGTGTAAGTGC	Pluripotency
NANOG	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG	Pluripotency
AP2α (TFAP2A)	TTCTGTTCAGTTCCGGGTCG	GCCACCGTGACCTTGACTT	Non-neural / mammary commitment
AP2γ (TFAP2C)	CGCTCATGTGACTCTCCTGA	GCATGTTCTTCCTAGCTGCC	Non-neural / mammary commitment
GATA3	TCATTAAGCCCAAGCGAAGG	GTCCCCATTGGCATTCTC	Non-neural / mammary commitment

Gene	Forward (5'→3')	Reverse (5'→3')	Functional category
TP63 (P63)	CAGGAAGACAGAGTGTGCTGGT	AATTGGACGGCGGTTCATCCCT	Non-neural / mammary commitment
TP63 (P63, alternate)	CTTGCCCAGGAAGAGACAGG	CATAAGTCTCACGGCCCCCTC	Non-neural / mammary commitment
PAX6	CTGAAGCGGAAGCTGCAAAG	TTGCTGGCCTGTCTTCTCTG	Neural ectoderm
TUBB3	GCGGATCAGCGTCTACTACA	GCCCCACTCTGACCAAAGAT	Neural ectoderm
OTX2	GAGTCGAGGGTGCAGCTATG	TGGCCACTTGTTCCACTCTC	Neural ectoderm
FOXP1	ACAGCTCCGTGTTGACTCAG	AGGGGTTGAGGGAGTAGGTC	Neural ectoderm
CK5 (KRT5)	CATGGACAACAACCGCAACC	ACTGCTACCTCCGGCAAGAC	Basal / myoepithelial
CK7 (KRT7)	AGGAGAGCGAGCAGATCAAG	CAGAGATATTCACGGCTCCC	Luminal
CK8 (KRT8)	TGGCAATCTGGGCTTGTAGG	AGAACGACATCCATGGGCTC	Luminal
ER α (ESR1)	CCACCAACCAGTGCACCATT	GGTCTTTTCGTATCCACCTTTC	Hormone receptor
ER β (ESR2)	AGAGTCCCTGGTGTGAAGCAA	GACAGCGCAGAAGTGAGCATC	Hormone receptor
PR (PGR)	CGCGCTCTACCCTGCACTC	TGAATCCGGCCTCAGGTAGTT	Hormone receptor

Gene	Forward (5'→3')	Reverse (5'→3')	Functional category
PRLR	CATGGTGACCTGCATCTTTCCG	GTGGGAGGAAAGTCTTGGCATC	Hormone receptor

Table 11. Primer panel used for end-point RT-PCR and qRT-PCR. Gene symbols follow HGNC nomenclature; synonyms employed in the thesis and in the original publications are given in parentheses. Two independent TP63 primer pairs were validated and used interchangeably depending on the experiment.

3.8. Immunofluorescence

Immunofluorescence (IF) was used to determine the subcellular localization of ER α and PR protein in undifferentiated iPSCs of the four reprogramming-origin lines. Cells were fixed and permeabilized with the Fix&Perm Reagent kit (Nordic-MUBio, Susteren, The Netherlands) according to the manufacturer's instructions, then blocked for 30 min with iBind™ Buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Primary antibodies were applied for 2 h at room temperature (RT): anti-ER (clone SP1, rabbit, ready-to-use; Ventana, Roche, Basel, Switzerland), which selectively recognizes the ER α isoform; anti-PR (clone 1E2, rabbit, ready-to-use; Ventana, Roche), which detects both PR-A and PR-B isoforms; anti-CD44 (clone DF1485, mouse, 1:50; Dako, Glostrup, Denmark), used to identify fibroblasts; and Alexa Fluor-488–phalloidin (1:500; Merck KGaA, Darmstadt, Germany) to visualize the actin cytoskeleton. Secondary antibodies (goat anti-mouse and anti-rabbit IgG (H+L) Alexa Fluor-568, 1:250; Thermo Fisher Scientific, Inc.) were co-incubated as appropriate. Nuclei were counterstained with DAPI for 5 min.

Images were acquired on an inverted fluorescence microscope (Olympus IX70, Olympus Optical Co. GmbH, Hamburg, Germany) at 10 $\times$ and 60 $\times$ magnification and analyzed with Image-Pro Plus software v7.0 (Media Cybernetics, Inc., Rockville, MD, USA). At least four independent fields were acquired per cell line across two independent staining experiments. A no-primary-antibody control was included in every experiment to establish the background fluorescence threshold.

3.9. Immunohistochemistry

The histological and immunohistochemical characterization of the mammary-like organoids was carried out in collaboration with the Pathological Anatomy Section of ASST Spedali Civili di

Brescia. Two sample-processing strategies were required to accommodate the different physical nature of the specimens.

Mature day 35 and day 20 organoids, already embedded in Matrigel/collagen domes, were fixed in situ in 4% formaldehyde for 24 h, transferred to labelled biocassettes, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin. Day 10 mammary embryoid bodies, which are too small to survive standard FFPE processing, were instead collected by centrifugation (500 rpm, 5 min) after fixation in 10% formalin for 24 h; the pellets were resuspended in a 1:2 mixture of plasma and HemosIL8 RecombiPlasTin 2G (Instrumentation Laboratory, Bedford, MA, USA) and allowed to clot, and the resulting clot was placed in a labelled cassette for paraffin embedding. In both cases, 2- μ m-thick sections were cut for hematoxylin–eosin (H&E) staining used to assess morphological integrity, and 2- or 4- μ m sections were cut for immunohistochemistry.

H&E staining followed the standard sequence of Mayer's hematoxylin (10 min) for nuclear contrast, rinsing in running water (2 min), acidified eosin (2 min) for cytoplasmic counterstaining, followed by dehydration through graded alcohols, clearing in xylene, and mounting with a coverslip.

For IHC, endogenous peroxidase activity was quenched during rehydration by incubation in methanol with 0.03% hydrogen peroxide for 20 min. Heat-mediated antigen retrieval was performed in a microwave oven (or water bath, when indicated by the antibody data sheet) using EDTA buffer at pH 8.0. The primary antibodies used, with their clones, dilutions and suppliers, are listed in Table 12. Note that the ER antibody directly recognizes the ER α isoform, whereas the PR antibody detects both PR-A and PR-B isoforms. Reactions were developed with Novolink Polymer (Leica Microsystems, Wetzlar, Germany) or Goat-on-Rodent HRP (Biocare), using 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) as chromogen, and sections were counterstained with Mayer's (Mayer's) hematoxylin. Slides were acquired under a light microscope for morphological and expression-pattern analysis.

Target	Clone	Species	Antigen retrieval	Dilution	Supplier (catalogue)
PAN-CK	MNF116	Mouse	EDTA pH 8.0	1:200	Dako, Glostrup, Denmark
CK5 / CK5/6	D5/16B4	Mouse	EDTA pH 8.0	Ready-to-use	Invitrogen / Ventana, Roche
CK7	OV-TL 12/30	Mouse	EDTA pH 8.0	Ready-to-use	Dako, Glostrup, Denmark
CK14	LL002	Mouse	EDTA pH 8.0	1:100	Leica
CK18	DC-10	Mouse	EDTA pH 8.0	1:250	Biocare
GATA3	L50-823	Mouse	EDTA pH 8.0	Ready-to-use	Cell Marque / Roche (cat. 760-4897)
TP63 (P63)	4A4 (also used as 4A4+Y4A3 cocktail)	Mouse	EDTA pH 8.0	Ready-to-use / 1:50	Roche / Cell Marque
SMA	1A4	Mouse	EDTA pH 8.0	Ready-to-use	Cell Marque
PR	1E2	Rabbit	EDTA pH 8.0	Ready-to-use	Ventana, Roche (cat. 790-2223)
ER (ER α)	SP1	Rabbit	EDTA pH 8.0	Ready-to-use	Ventana, Roche

Table 12. Primary antibodies used for immunohistochemical characterization of mammary embryoid bodies and mammary-like organoids. The ER antibody specifically recognizes the ER α isoform; the PR antibody recognizes both PR-A and PR-B isoforms.

3.10. Statistical analysis

All statistical analyses were performed with GraphPad Prism v8.0 (GraphPad Software, Inc., San Diego, CA, USA). Numerical data are expressed as mean $\pm$ standard error of the mean (SEM) and were obtained from at least three independent biological replicates, each analyzed in technical triplicate. Differences among multiple experimental groups (e.g. across iPSC lines, or across differentiation time points) were assessed by one-way ANOVA followed by Tukey's post-hoc test, whereas pairwise comparisons between a single experimental condition and its control were assessed by unpaired two-tailed Student's t-test. Differences were considered statistically significant when $p \leq 0.05$. Significance levels are indicated in the figures as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Chapter 4

Results

4.1. Characterization of the Episomal iPSC Line

The Episomal Gibco® iPSC line (Thermo Fisher Scientific), reprogrammed from CD34⁺ cord blood progenitors using episomal vectors expressing seven factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, and SV40 T), was used throughout this study.

4.1.1. Morphological Characteristics

iPSC colonies appeared as tightly packed, well-circumscribed, rounded aggregates with smooth edges and a high nucleus-to-cytoplasm ratio (**Figure 1**). Cells within the colonies had large nucleoli and were morphologically uniform, reflecting a homogeneous, undifferentiated cell population. Cells were plated onto Matrigel-coated plates for expansion and cultured in NutriStem® hPSC XF Medium, with bFGF (basic Fibroblast Growth Factor) added daily to maintain an undifferentiated status.

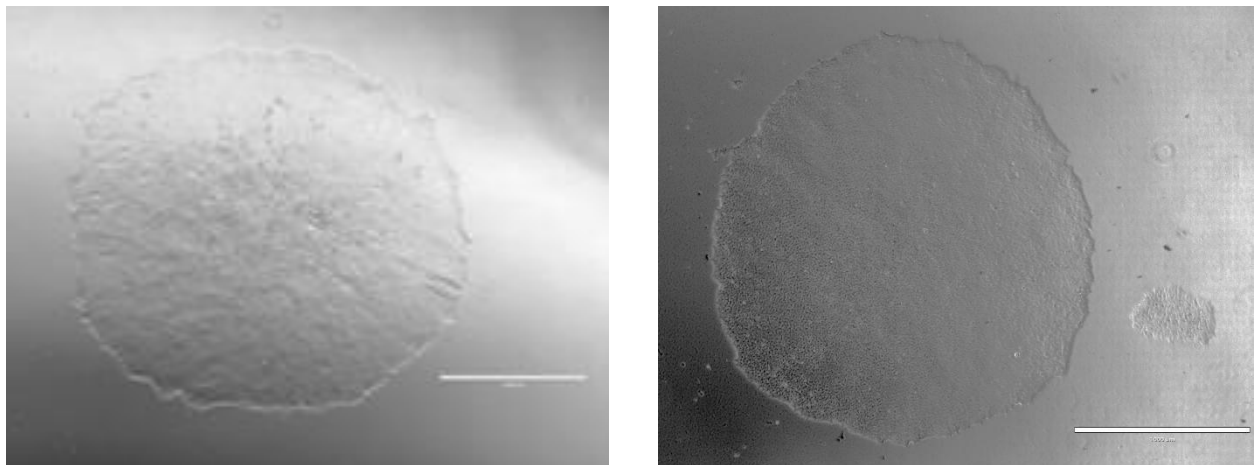


Figure 1: iPSC colonies: Episomal iPSC colony at passage 120 is shown at 4x magnification.

4.1.2. Quality Control: Pluripotency Marker Verification

As the cell line is commercially available, comprehensive characterization is not strictly necessary. Nevertheless, before initiating differentiation, it is advisable to confirm stemness and pluripotency, including morphological features and marker expression, under the specific culture conditions used.

RT-PCR confirmed consistent transcriptional activity of NANOG, OCT4, SOX2, and KLF4 across successive passages. The presence of these markers at each passage indicated that the episomal iPSC line retained its pluripotent character without evidence of spontaneous differentiation or progressive silencing of the reprogramming transgenes. Periodic monitoring of culture supernatants ruled out mycoplasma contamination throughout the extended culture period. (Figure 2)

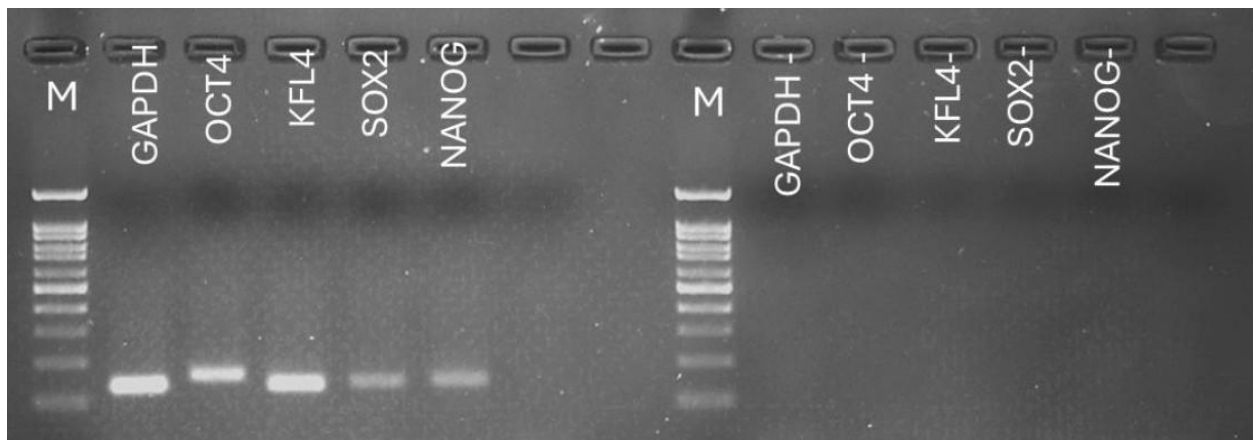


Figure 2: Gel electrophoresis confirming expression of OCT4, KLF4, SOX2 and NANOG (GAPDH as reference) in the Episomal iPSC line. Lanes 2–6: iPSC cDNA. Lanes 10–14: culture supernatant, showing no amplification. Lanes 7 and 15 are negative controls.

4.2. Molecular Profiling of Mammary Embryoid Bodies and Initial Detection of Progesterone Receptor

4.2.1. Generation of Mammary Embryoid Bodies (mEBs)

Induced pluripotent stem cells (iPSCs) were unenzymatically dissociated using Gentle Cell Dissociation Reagent (STEMCELL Technologies). A total of 1.2×10^6 cells were seeded into each well of an AggreWell™ culture plate containing 300 microwells per well, corresponding to approximately 4,000 cells per microwell. The plates were pre-treated with an anti-adhesion coating, and centrifugation was performed to facilitate rapid and uniform cell aggregation. This controlled aggregation approach generated approximately 300 embryoid bodies per well, all comparable in size and morphology, fundamental conditions for reducing experimental variability.

Microscopic imaging at 24 hours confirmed the formation of compact, uniform spheroids (**Figure 3**). Before proceeding to the next culture phase, careful visual selection was performed: only single, non-fused spheroids were retained for further differentiation, while any fused or irregularly shaped aggregates were excluded to ensure morphological homogeneity within the starting population.

The selected mEBs were then transferred to low-adhesion culture plates and maintained for ten days in MammoCult™ medium (STEMCELL Technologies), with complete medium replacement every 48 hours. The low-adhesion surface prevented cell attachment and preserved the three-dimensional architecture of the mEBs throughout this period—a condition essential for proper lineage specification. MammoCult™ medium, specifically formulated for mammosphere growth, has been shown to direct embryoid bodies toward a non-neural ectodermal fate, suppressing the predominance of neural ectoderm while promoting the emergence of the progenitor subpopulation from which basal and luminal mammary cells ultimately arise.

At the end of the ten-day, the mEBs were embedded in dome-shaped three-dimensional matrices composed of Matrigel and Collagen I. This time point was selected because day 10 mEBs represent an optimal stage for matrix incorporation and subsequent organoid maturation, with peak enrichment of non-neural ectodermal features.

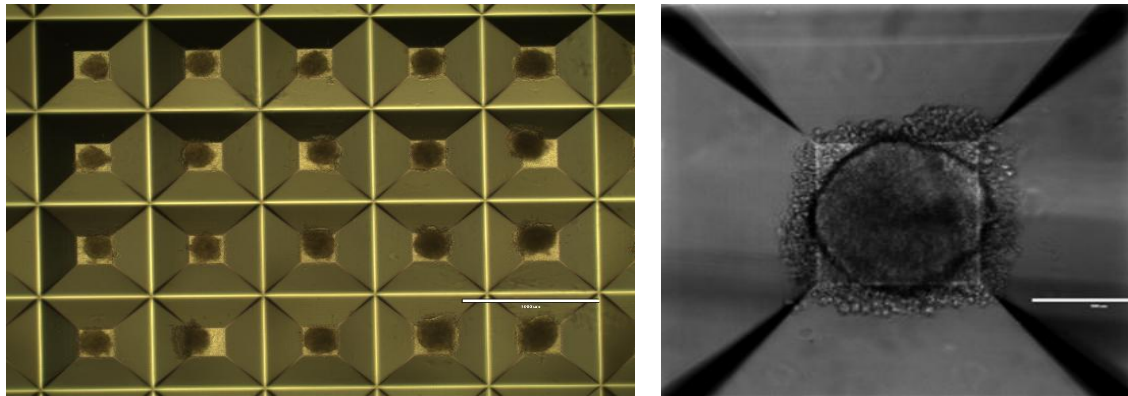


Figure 3: EBs derived from iPSCs of the episomal cell line after 24 h, shown at 4× and 20×magnification. The 4× image shows the microwell plate overview, and the 20× image shows a single EB in one microwell.

4.2.2. Gene Expression Associated with Non-Neural Ectoderm Induction Over Days (0–10)

To monitor the molecular transition from pluripotency toward a mammary-competent progenitor state during the ten-day culture described in section 4.2.1, qRT-PCR was performed on cell samples collected at days 0, 2, 5, and 10 (**Figure 4**).

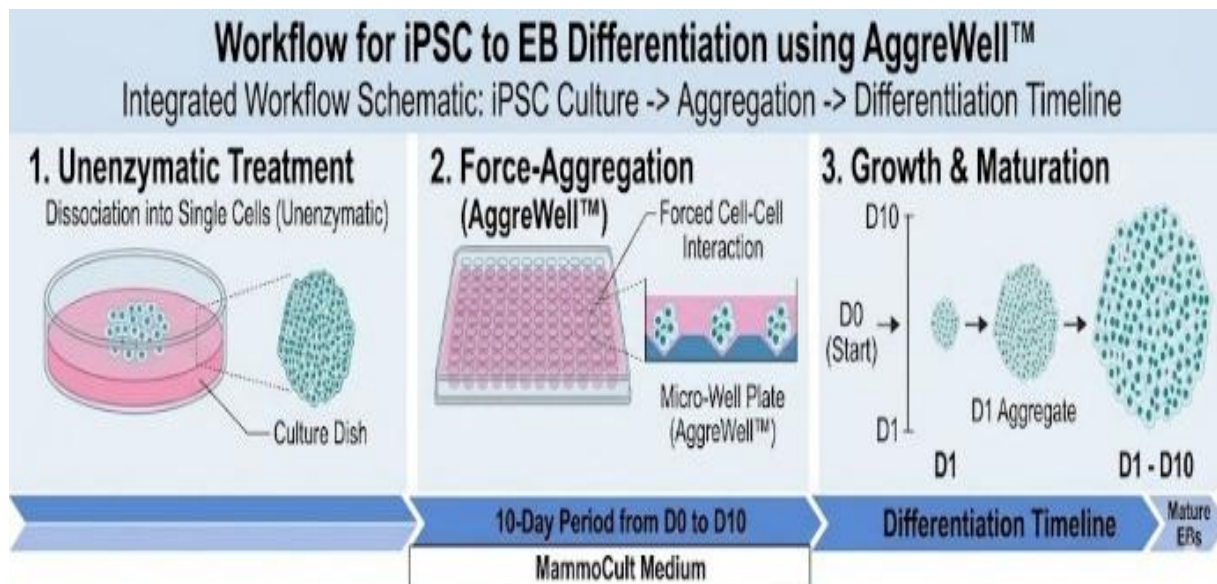


Figure 4: Schematic representation of the EB generation procedure.

Expression was assessed for a panel of markers grouped into four functional categories:

Pluripotency markers – OCT4 and SOX2: highly expressed in undifferentiated iPSCs and early embryoid bodies; their progressive decline indicates loss of the stem cell state and onset of differentiation.

- **Non-neural ectoderm / mammary commitment markers** – P63 (essential for basal cell development), GATA3 (key regulator of luminal cell differentiation), AP2 α and AP2 γ (involved in epithelial development and mammary lineage specification), and the progesterone receptor (PR).
- **Neural ectoderm markers** – PAX6, TUBB3, OTX2, and FOXG1: typically expressed in neural progenitor cells; their expected decrease would reflect successful suppression of the neural fate.
- **Early epithelial marker** – CK8 (a keratin associated with luminal epithelial differentiation).

Day 0 expression values, derived from undifferentiated iPSCs prior to embryoid body formation, served as baseline for all subsequent time-point comparisons.

4.2.3. Gene Expression Analysis by qRT-PCR

Figures 5 and 6 present relative quantification (RQ) values at each time point, calculated by the $2^{-\Delta\Delta CT}$ method and normalized to day 0 iPSC gene expression, which was used as the calibrator.

Pluripotency markers – OCT4 and SOX2 were elevated on day 0 and progressively decreased through day 10, confirming loss of pluripotency and initiation of differentiation.

Non-neural ectoderm / mammary commitment markers Non-neural ectoderm/mammary commitment markers—P63, GATA3, and AP2 α —showed a progressive increase in expression over the 10 days, reaching peak levels on day 10 compared with day 0. This trend is consistent with the enrichment of a non-neural/progenitor population capable of generating basal and luminal mammary components. AP2 γ followed a similar pattern, while PR expression was detectable but remained relatively low during this early phase.

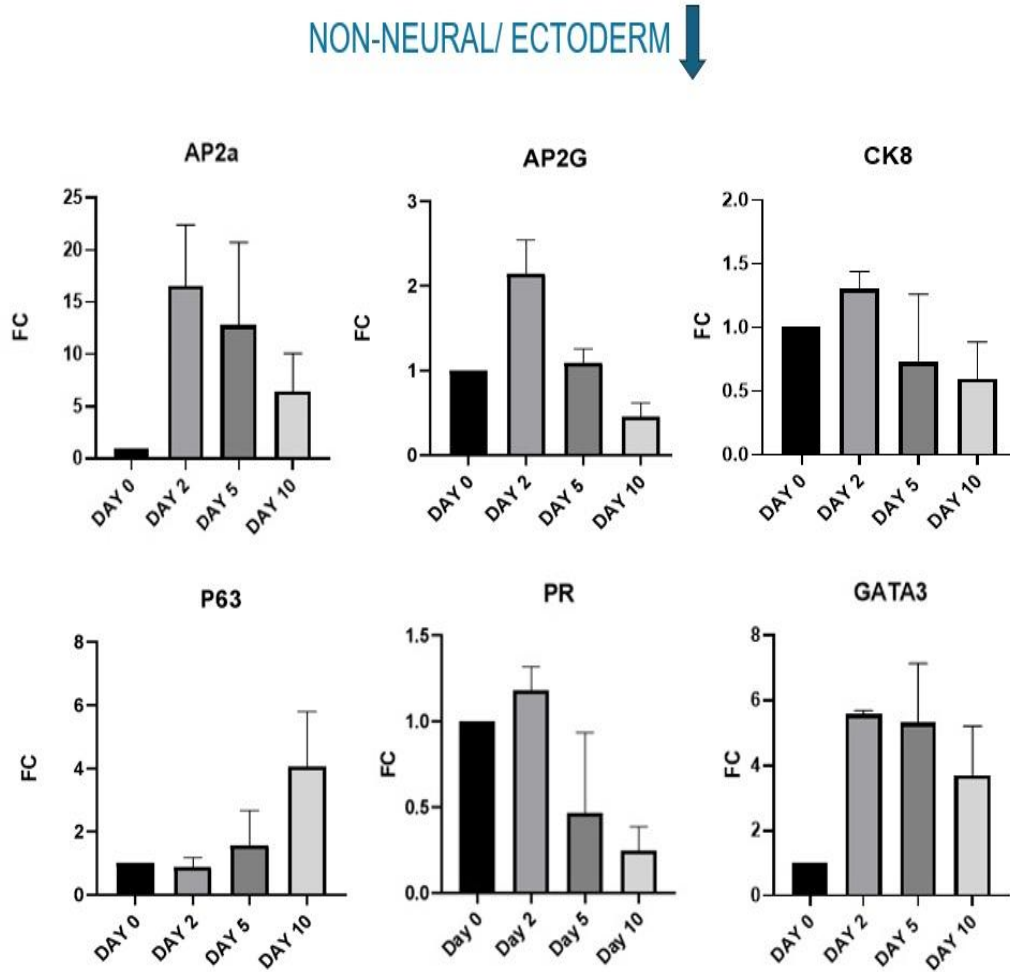


Figure 5: qPCR analysis of non-neural ectoderm markers associated with mammary commitment in mEBs samples.

Early epithelial marker – CK8 showed a significant increase starting on day 2, with a slightly lower level on day 10. This increase is consistent with the acquisition of a luminal epithelial phenotype and with progressive mammary commitment at the transcriptional level.

Neural ectoderm markers – TUBB3 decreased as early as the first few days, consistent with suppression of the neural fate. However, PAX6 exhibited an upward trend at certain times, contrary to expectations. FOXG1 expression was low on days 2 and 5 but increased by day 10. These findings indicate that, although the non-neural program is predominant, residual heterogeneity in the mEBs population may allow persistence or late emergence of some neural signals.

the qPCR results delineate a picture consistent with the experimental goal: loss of pluripotency, enrichment of non-neural ectoderm markers (P63, GATA3, AP2 α), and acquisition of epithelial traits (CK8). Nevertheless, the late or unexpected expression of some neural markers (PAX6,

FOXC1, OTX2) indicates that the mEBs population remains heterogeneous at day 10, a caveat that should be considered before matrix embedding.

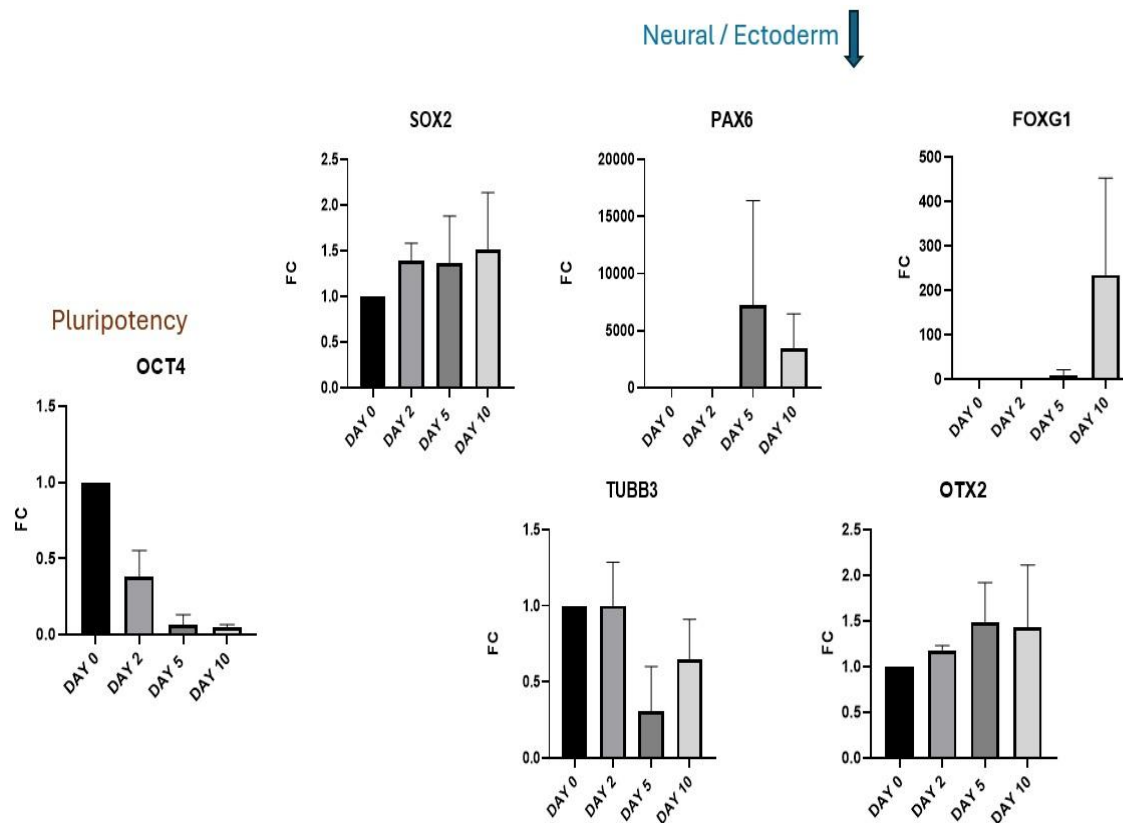


Figure 6: qPCR analysis of pluripotency and neural ectoderm markers in mEBs samples

4.2.4. Organoid Maturation and Branchio-Alveolar Induction Through Day 35

Following matrix embedding on day 10 (section 4.2.1), the dome cultures were maintained for an initial five days in EpiCult-B medium supplemented with parathyroid hormone-related protein (PTHrP) to promote mammary epithelial commitment. This phase preceded the onset of branchio-alveolar induction and served to expand both bipotent and lineage-restricted luminal and myoepithelial progenitors within the developing organoid structures.

On day 15, the medium was replaced with EpiCult-B supplemented with hydrocortisone, FGF10, insulin, and HGF to induce branching morphogenesis and further epithelial differentiation. The medium was subsequently replaced every three days until day 35. Over this period, a cluster of

epithelial cells emerged from the central body of each organoid and gradually organized into branched structures with an internal luminal component. Representative images of the same organoid at day 15 (**A**), day 35 (**B**), revealed the progressive formation of branched structures within the Matrigel, consistent with a rudimentary ductal tree (**Figure 7**).

Hypothetically, epithelial acinar maturation could be further completed by inducing lactational differentiation through the addition of prolactin to EpiCult-B.

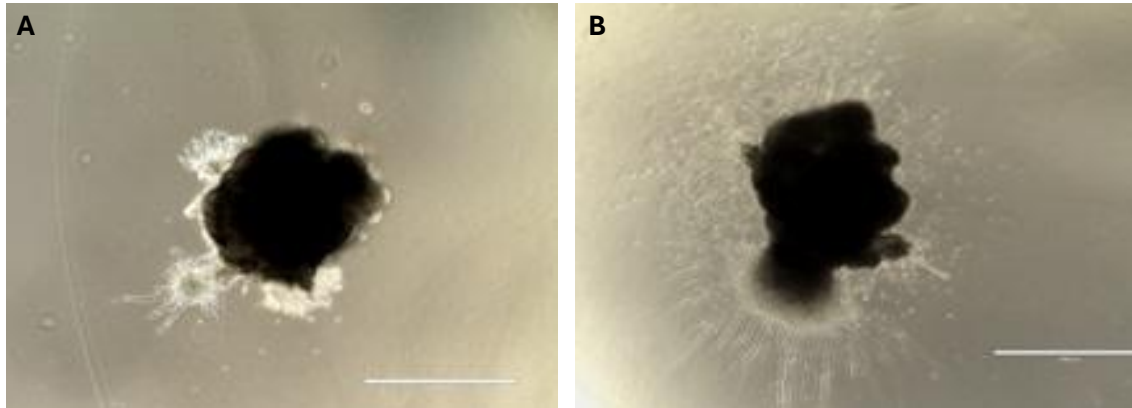


Figure 7: Dome morphology at day 15 (A), day 35 (B)

4.2.5. Prolactin Receptor Expression During Organoid Maturation in Dome Culture

To assess whether the maturing organoids acquired the molecular competence required for lactogenic differentiation, prolactin receptor (PRLR) gene expression was evaluated by qRT-PCR in organoids collected from dome culture at day 5 and day 20. In organoids harvested on day 5, PRLR expression remained at baseline levels, whereas during the differentiation stage from day 5 to day 20, PRLR was progressively upregulated, reaching substantially higher levels in organoids collected on day 20 (**Figure 8**). This increase indicates that, as organoids matured within the Matrigel dome, they progressively acquired prolactin receptor expression — a prerequisite for the lobuloalveolar remodeling that occurs during pregnancy and lactation *in vivo*. The temporal upregulation of PRLR transcript levels provided the rationale for initiating the lactogenic phase on

day 35 by prolactin supplementation, as the organoids within the dome had developed sufficient receptor expression to respond to prolactin signaling.

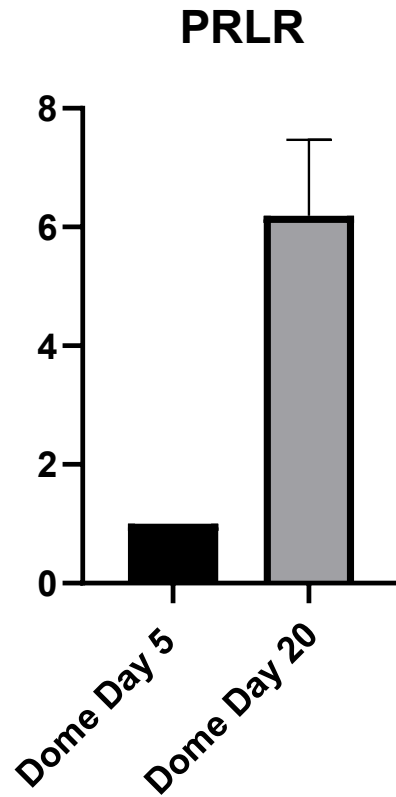


Figure 8: PRLR expression in organoids at dome day 5 and dome day 20 by qRT-PCR. Error bars: $\pm$ SEM

4.2.6. Histological Characterization of *In Vitro*-Generated Breast Organoids at Day 35

The H&E stain of cross-sectioned organoids on day 35 (**Figure 9**) confirms epithelial architecture equivalent to that of the mammary gland, with blue-stained epithelial nuclei and pink-stained surrounding matrix. The defined luminal spaces and stroma show the segmented, organized structure of the epithelial cells. This histology serves as a reference for both the morphology of the organoid cultures and for interpreting immunohistochemical staining of markers for luminal and basal mammary epithelial cells.

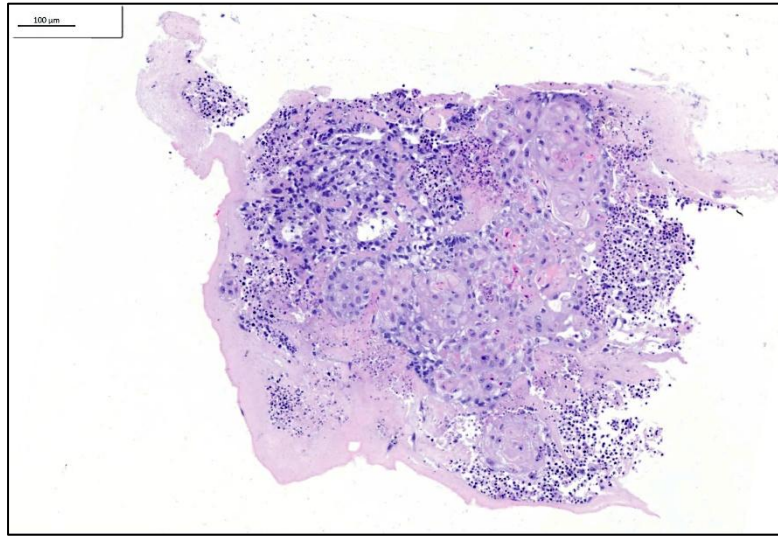


Figure 9: Histological section of a fully differentiated mammary organoid embedded in paraffin and stained with hematoxylin and eosin (H&E), showing cellular components and extracellular matrix.

Immunostaining for CK5/6 (**Figure 10**) revealed strong positivity localized to cells at the periphery of ductal and alveolar structures. CK5/6 are high-molecular-weight cytokeratin characteristic of basal and myoepithelial cells of the mammary gland. The observed positivity indicated the presence of a basal progenitor population and differentiated myoepithelial cells, suggesting the development of the cellular hierarchy typical of mammary gland tissue.

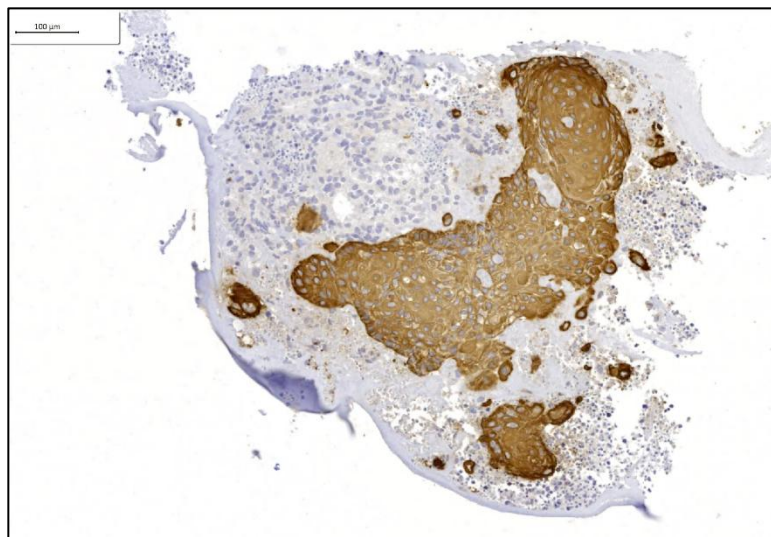


Figure10: Histological section of a fully differentiated mammary organoid immunolabeled with anti-CK5/6 to identify basal and myoepithelial progenitor cells.

CK14 staining (**Figure 11**) showed that the CK14 staining pattern was representative of a regional distribution pattern (not diffuse) where CK14 (brown) was found in certain distinct cellular

compartments of the organoid structure and showed the presence of basal cells (supporting ductal or alveolar structures). CK14 expression confirmed that the organoids contained basal cells (with a distinct basal compartment), mimicking the bilayer organizational pattern seen in normal mammary glands *in vivo*.

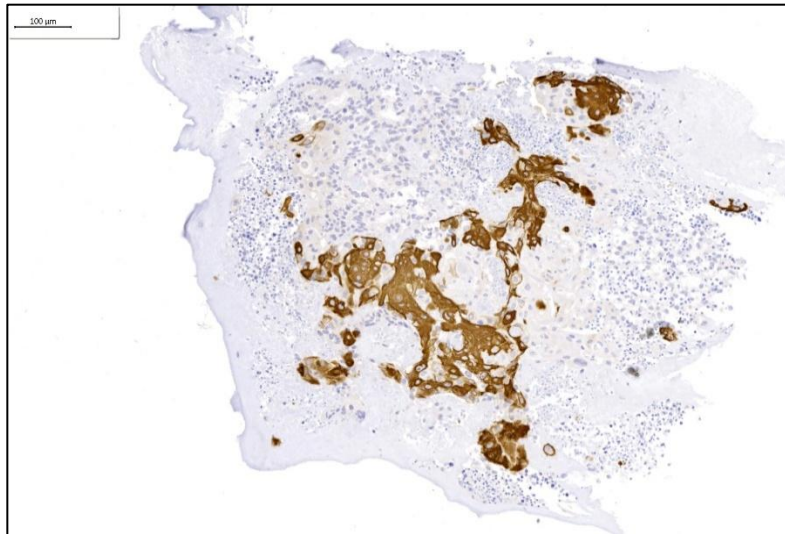


Figure 11: Histological section of a fully differentiated paraffin-embedded mammary organoid stained with anti-CK14 antibody to identify basal cell populations.

Moderate positivity for CK18 (**Figure 12**), a marker of luminal epithelial identity, was detected within the internal structures of the organoids, which are thought to represent ducts or alveoli. The significant presence of CK18 indicated that commitment of the mammary epithelial cells toward the luminal lineage was well underway.

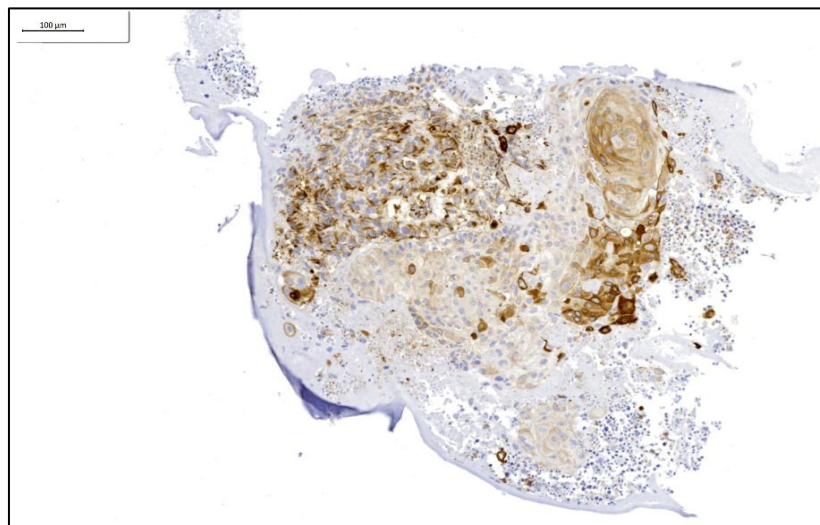


Figure 12: Histological section of a fully differentiated mammary organoid stained with anti-CK18, highlighting luminal ductal cell populations within the organoid.

GATA3 positivity was observed in luminal epithelial cells lining the alveoli and ducts (**Figure 13**), with nuclear staining evident in the luminal compartment. This pattern indicated that correct luminal differentiation had occurred, suggesting the development of secretory functionality within the organoid.

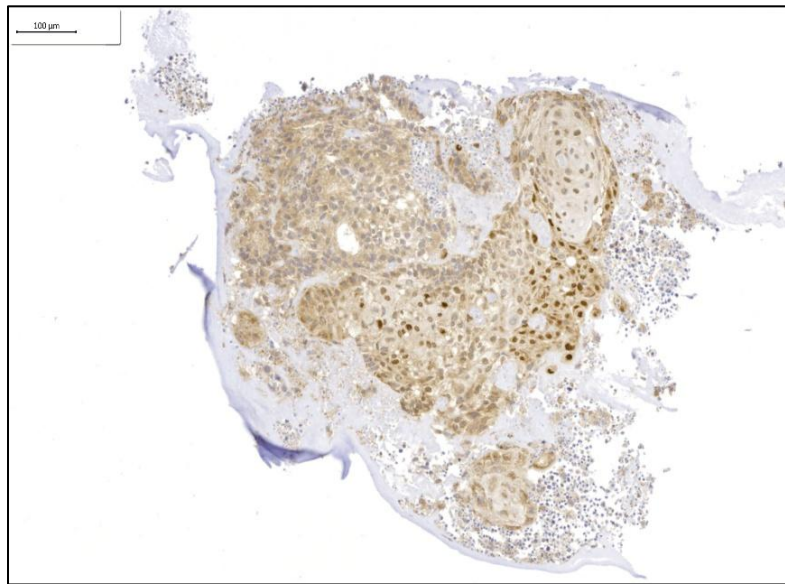


Figure 13: Histological section of a fully differentiated mammary organoid immunolabelled with anti-GATA3, highlighting luminal epithelial cells lining the ductal structures.

α -smooth muscle actin (SMA) positivity (**Figure 14**) confirmed the differentiation of myoepithelial cells forming the outer basal layer surrounding the ducts and alveoli. SMA provides structural support to the ductal network and contributes to the contraction necessary for milk ejection. The localization of SMA-positive cells around the luminal structures demonstrated that the organoid maintained the characteristic bilayered architecture of the mammary gland, comprising both the luminal and myoepithelial compartments.

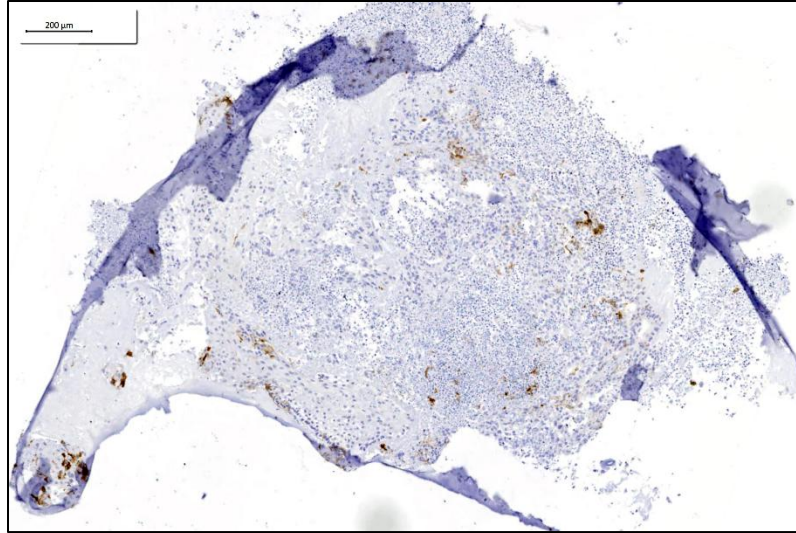


Figure 14: Histological section of a fully differentiated mammary organoid stained with anti-SMA, highlighting basal cells surrounding the ducts and providing structural support.

The extensive nuclear positivity of the progesterone receptor (PR) immunostaining (**Figure 15**) was used to evaluate hormone responsiveness of epithelial cells, as well as to identify how much progesterone could stimulate proliferation, differentiation, and tissue organization. The widespread presence of PR positive nuclei suggests there was a population of cells that had completed maturation to become a hormonally responsive population of mature luminal cells.

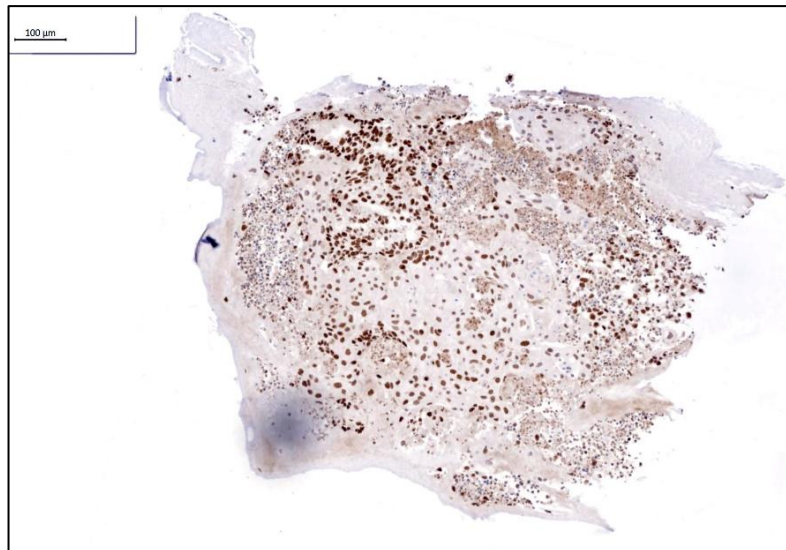


Figure 15: Histological section of a fully differentiated mammary organoid immunolabelled with anti-PR antibody, showing PR-expressing luminal epithelial cells within the organoid.

P63 immunostaining (**Figure 16**) revealed discrete cellular compartments positive for this basal progenitor transcription factor. P63 is expressed by myoepithelial and basal cells located near the ducts and alveoli, and its detection is important for confirming the maintenance of the basal cell population within the organoid model.

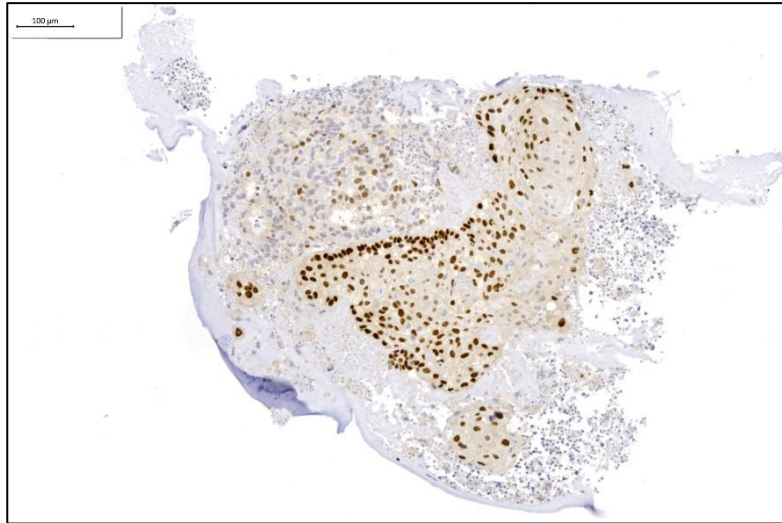


Figure 16: Histological section of a fully differentiated mammary organoid immunolabelled with anti-p63 to identify basal progenitor cell populations within the organoid.

The absence of nuclear positivity for estrogen receptor (ER) immunostaining (**Figure 17**) was used to indicate the lack of hormone responsiveness in the epithelial cells, as well as to assess the limited involvement of estrogen signaling in promoting proliferation, differentiation, and tissue organization at this stage. The lack of ER-positive nuclei suggests that the cell population had not acquired the characteristics of a hormonally responsive mature luminal phenotype.

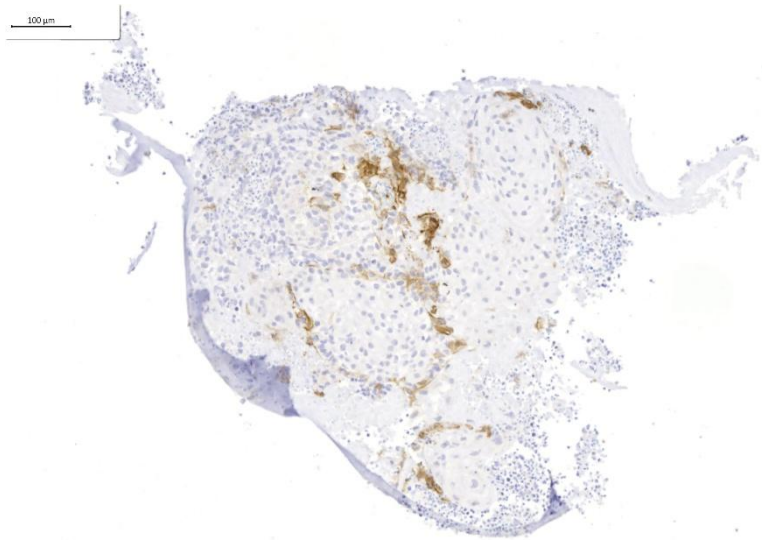


Figure 17: Histological section of a fully differentiated mammary organoid immunolabelled with anti-ER antibody, showing lack of ER-expressing luminal epithelial cells within the organoid

4.3. Assessment of Terminal Differentiation Marker Gene Expression

Given that lactogenesis induction in mammary organoids remains challenging across different cellular origins (Sumbal et al., 2020), the expression of prolactin receptor (PRLR) was evaluated as a preliminary indicator of lactogenic potential in two selected organoids, named A8 and B6 (J. Lee et al., 2023). Two established breast cancer cell lines were included as expression controls: MCF-7, which expresses both PRLR and progesterone receptor and is widely used in PRLR signaling studies (Ogawa et al., 2003), and MDA-MB-231, which lacks both PR and ER expression.

RT-PCR analysis revealed distinct PRLR expression in both end-stage organoids (A8 and B6), while only a faint band was observed in day 10 mEBs (**Figure 18**).

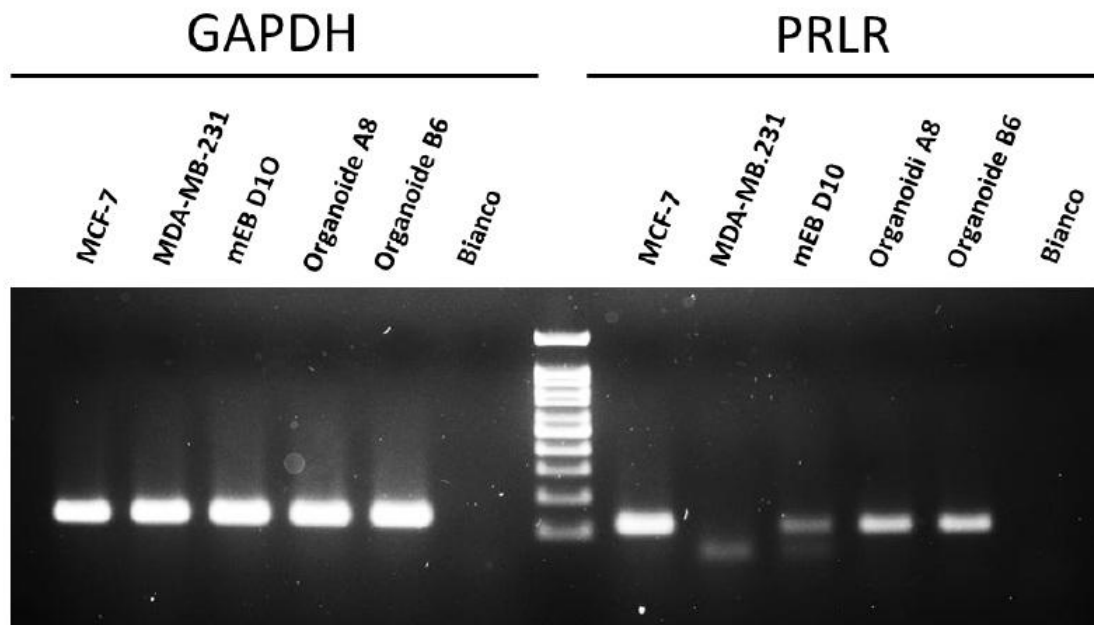


Figure 18: RT-PCR analysis of prolactin receptor (PRLR) expression using GAPDH as an internal control in MCF-7 (positive control), MDA-MB (negative control), D10, and dome (A8, B6) samples.

PR expression was subsequently assessed in the same samples and was detected in both mature organoids (**Figure 19**). Together, these findings confirmed at the transcriptional level that the differentiated structures had acquired a hormone-responsive luminal epithelial phenotype.

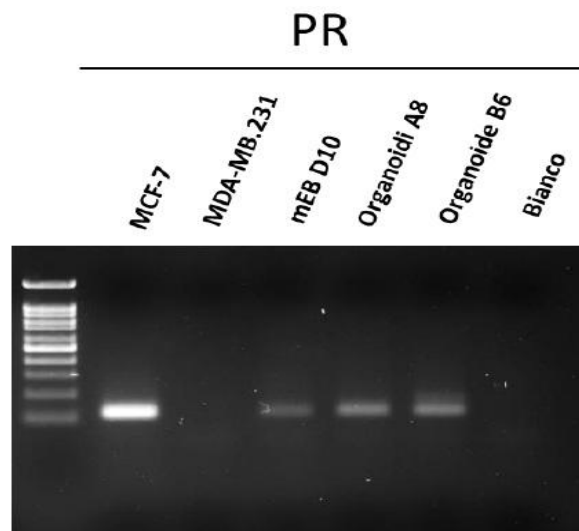


Figure 19: RT-PCR analysis of progesterone receptor (PR) expression in MCF-7 (positive control), MDA-MB (negative control), and samples D10 and dome (A8, B6).

4.4. Complementary Investigation of Steroid Hormone Receptor Expression Across Multiple iPSC Lines

Given the consistent absence of estrogen receptor α (ER) protein during early differentiation attempts, we reasoned that a broader, stage-resolved analysis of both ER and progesterone receptor (PR) expression was required to understand whether hormone responsiveness might emerge later in the mammary differentiation trajectory. We therefore designed a systematic time-course experiment, collecting samples at key transitions: undifferentiated iPSCs, day 10 mammary embryoid bodies, and day 20 mammary-like organoids. This approach allowed us to profile ER α , ER β , and PR at both the transcript and protein levels across the entire differentiation process.

4.4.1. Transcriptional Profiling of Estrogen and Progesterone Receptors in iPSC Lines

The expression of ER α , ER β , and PR transcripts was evaluated in four independent iPSC lines. **(Figure 20)** compared to MCF7 (ER α^+ /ER β^- /PR $^+$) and MDA-MB-231 (ER α^- /ER β^+ /PR $^-$) human breast cancer cell lines. The four lines represented two distinct somatic cell types and four different reprogramming technologies: Episomal (CD34 $^+$ -derived, episomal vectors), BJ (fibroblast-derived, Sendai virus), 253-G1 (fibroblast-derived, retrovirus), and F3 (fibroblast-derived, lentivirus).

Quantitative PCR analysis showed that ER α mRNA levels were significantly lower across all iPSC lines compared with the MCF7 positive control, indicating markedly reduced ER α transcript abundance in these cells. No significant differences in ER α expression were detected among the iPSC lines. Similarly, PR mRNA levels were significantly reduced in iPSCs compared to MCF7 cells, and this reduction was consistent across all lines, regardless of origin or reprogramming technique. Thus, the iPSCs consistently showed low but detectable PR mRNA.

iPSCs F3 expressed the ER β at levels comparable to the ER β -positive MDA-MB-231 control, with significant upregulation relative to the ER β -negative MCF7 line. Other iPSC lines showed only a trend toward higher the ER β expression. These results indicate that undifferentiated iPSCs do not actively transcribe the ER α , express low but detectable levels of the PR, and exhibit variable, line-specific upregulation of the ER β , most pronounced in iPSCs F3.

These results indicate that iPSCs do not actively transcribe ER α , whereas PR mRNA is expressed at low levels and ER β is actively transcribed.

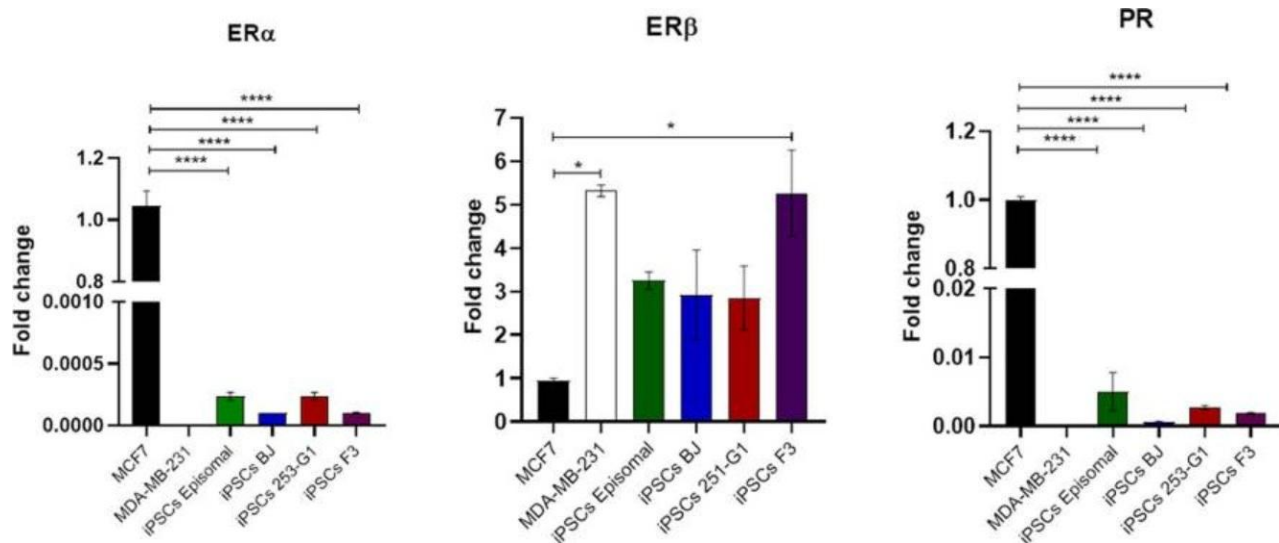


Figure 20: ER α , ER β , and PR mRNA expression in iPSC lines relative to MCF7 and MDA-MB-231 controls. (A) ER α , (B) ER β , and (C) PR expression levels. Histograms show fold change from three independent experiments; error bars indicate $\pm$ SEM.

4.4.2 Protein Expression and Subcellular Localization of ER α and PR in iPSCs

1. Localization of ER α and PR in iPSCs

Because transcript levels do not always correlate with protein abundance, we extended our analysis to the protein level by performing immunofluorescence on the different iPSC lines (**Figures 21 and 22**). The antibodies utilized in this study are those commonly applied in diagnostic microscopy, as described comprehensively in the Materials and Methods section. Notably, the antibody against the estrogen receptor specifically targets the ER α isoform, whereas the antibody for the progesterone receptor can detect both the A and B isoforms.

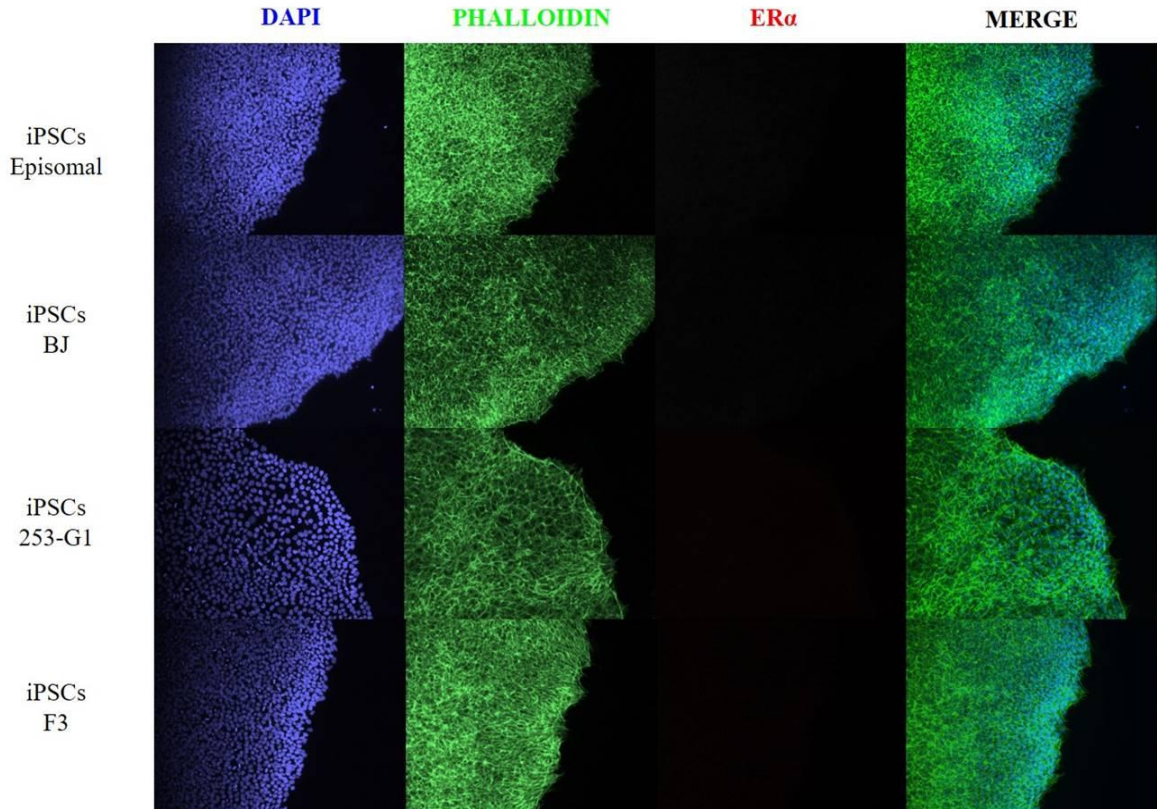


Figure 21:ER α detection in iPSC cell lines by immunofluorescence. Nuclei were counterstained with DAPI (blue), cytoskeleton with phalloidin-488 (green), and ER α with Alexa-568 (red). Magnification: 10 $\times$.

As shown in **Figure 21**, ER α protein was undetectable in all four iPSC lines examined (iPSCs Episomal, BJ, 253-G1, and F3). The red fluorescence channel signal corresponding to ER α staining was absent in both nuclear and cytoplasmic compartments across all imaging fields examined and was indistinguishable from the no-primary-antibody negative control background. These protein-level findings are concordant with the qPCR data.

Unexpectedly, as shown in **Figure 22**, we detected the expression of PR protein in the nucleus of all the iPSC lines generated from different parental cells with several reprogramming methods. PR protein was clearly and consistently detected in the nuclei of iPSCs Episomal, BJ, 253-G1, and F3, irrespective of somatic cell origin or reprogramming technology. Strong and specifically nuclear PR immunofluorescence staining was observed. The signal was reproducibly present across multiple independent imaging fields in at least two independent staining experiments per cell line. The staining was not diffusely distributed throughout the cytoplasm, confirming the specific

nuclear localization of PR protein in undifferentiated iPSCs. Taken together, these findings indicate that active translation of the progesterone receptor occurs in these cells.

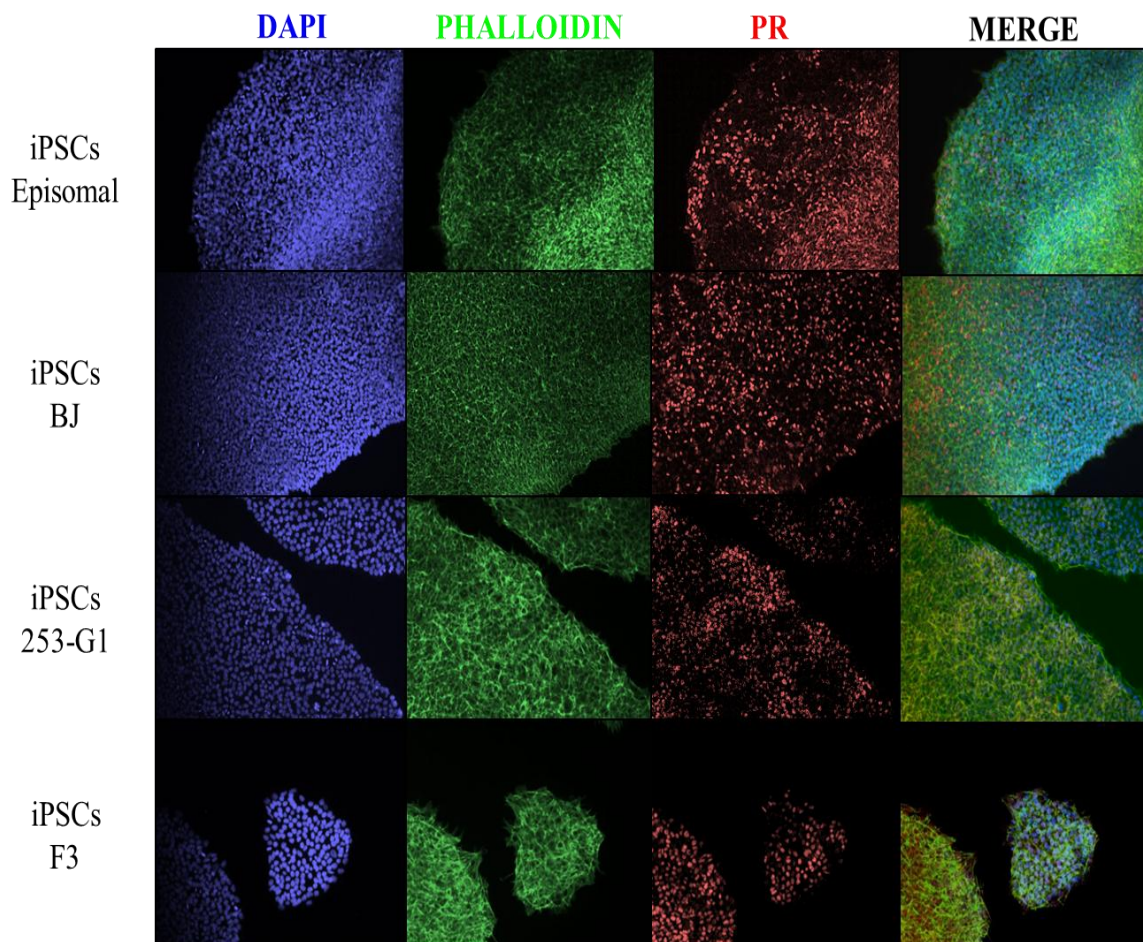


Figure 22: Immunofluorescence detection of PR in different iPSC lines. Nuclei were stained with DAPI (blue), cytoskeleton with phalloidin-488 (green), and PR with Alexa-568 (red). Images were acquired at 10× and 60× magnification. Representative images from at least four independent fields across two independent experiments are shown.

4.5. Generation and Morphological Characterization of Mammary-like Organoids from Multiple iPSC Lines

To understand whether PR expression may have functional implications during iPSC differentiation, mammary-like organoids were generated from two representative iPSC lines: iPSCs Episomal (CD34⁺ cord blood-derived, episomal reprogramming) and iPSCs BJ (fibroblast-

derived, Sendai virus reprogramming) to determine whether PR expression is maintained during directed differentiation toward the mammary epithelial lineage.

4.5.1. Morphological Progression During Differentiation

For both lines, mEBs generation, and three-dimensional matrix embedding were carried out following the procedure described in section 4.2.1. The morphological progression of differentiating cultures was monitored by phase-contrast microscopy at sequential stages (**Figure 23**).

Following aggregation, cells from both lines condensed into uniform spheroidal mEBs with well-defined boundaries by day one (**Figure 23B**). Over the subsequent ten days, these structures gradually increased in architectural complexity, with visible cellular reorganization at the aggregate peripheries (**Figure 23C**). After matrix embedding at day ten, progressive branching morphogenesis became increasingly evident: cellular extensions emerged from the central body of each organoid and extended into the surrounding matrix. By day twenty, the structures had developed a complex ductal and alveolar architecture featuring branched networks integrated within the gel (**Figure 23D**).

Importantly, these morphological outcomes—from initial spheroid formation through branching to ductal-alveolar organization—were consistently observed across both iPSC lines, confirming that the differentiation protocol is reproducible regardless of the somatic cell source or reprogramming technology employed.

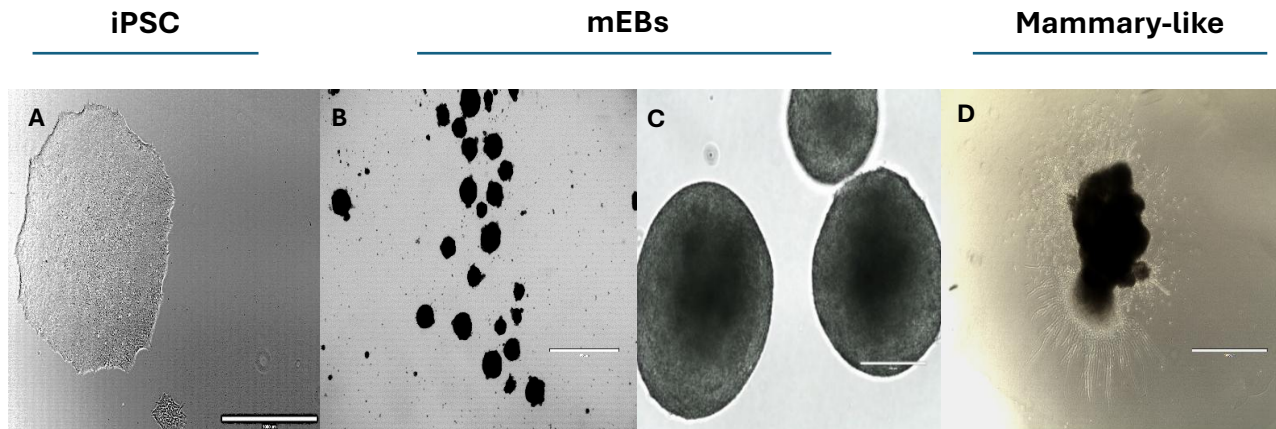


Figure 23: Representative morphological changes during the generation of mammary-like organoids from iPSCs: (A) iPSC colonies, (B) day 1 mEBs differentiation, (C) day 10 mEBs differentiation, and (D) mammary-like organoids. Images were acquired at 4× magnification.

4.6. Immunohistochemical and Transcriptional Validation of Mammary Epithelial Identity and Hormone Receptor Expression

To validate the mammary epithelial identity of the generated organoids and to characterize hormone receptor expression during differentiation, immunohistochemical (IHC) staining and longitudinal qRT-PCR analyses were performed across three consecutive stages: undifferentiated iPSCs (day 0), day 10 mammary embryoid bodies (mEBs), and day 20 mammary-like organoids (**Figure 24**).

4.6.1 Confirmation of Bilayered Mammary Epithelial Architecture

The presence of both luminal and basal epithelial compartments was confirmed by IHC staining of day 20 organoid sections, replicating the bilayered architecture of the native mammary gland (**Figure 24 B–G**). Luminal epithelial identity was established by positive staining for CK7, CK18, and GATA3, whereas basal and myoepithelial compartments were confirmed by positive staining for TP63 and CK5. The successful generation of mammary-like organoids was demonstrated by the observation of these markers in spatially distinct but coexisting populations within the same organoid structures.

Quantitative gene expression analysis across the differentiation time course supported these protein-based findings. CK5 expression remained very low in undifferentiated iPSCs and day 10 mEBs but increased dramatically in day 20 organoids; TP63 was similarly upregulated at the organoid stage. Among luminal markers, CK7 and GATA3 were progressively upregulated during differentiation, whereas CK18 expression remained unchanged across all three stages (**Figure 25**).

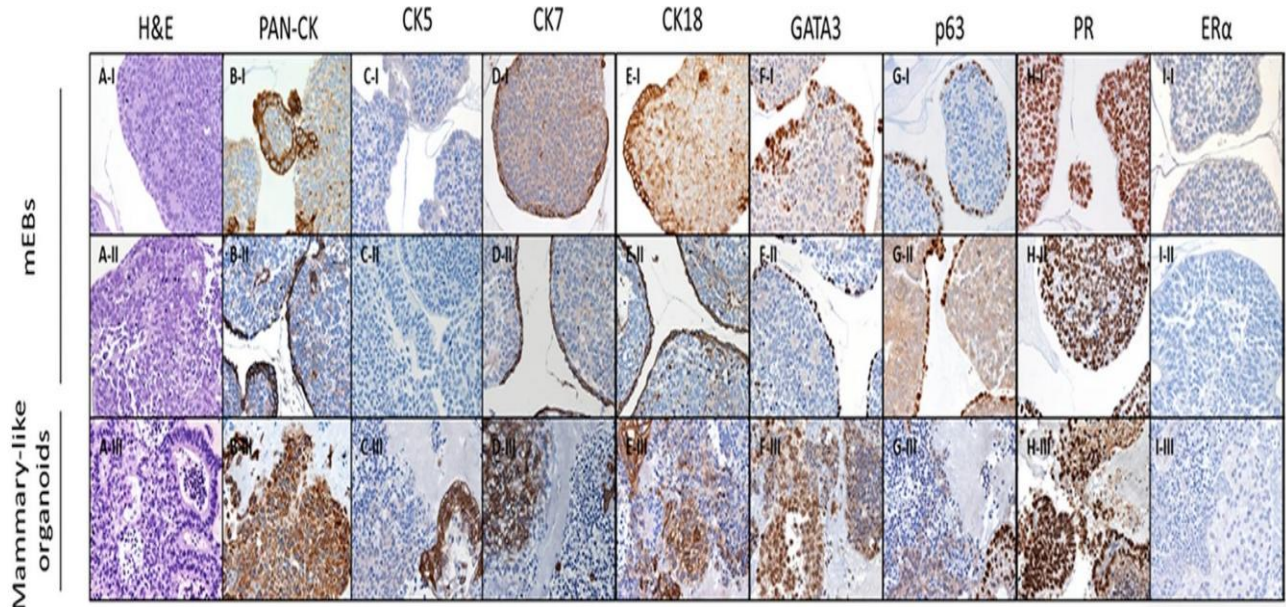


Figure 24: H&E and IHC staining. A) H&E stain; B) PAN-CK; C-D-F) Luminal (CK5/7 + and GATA3 +) and E-G) basal cells markers (CK18 + and TP63 +); H) PR and I) ERα. Representative images of mEBs obtained from iPSCs Episomal (I); mEBs obtained from iPSCs BJ (II); mammary-like organoids (III) differentiation

4.6.2. Hormone Receptor Expression During Differentiation

Immunohistochemistry (IHC) revealed positive nuclear progesterone receptor (PR) staining in day 20 mammary-like organoids, specifically localized to the luminal epithelial cell population (see **Figure 24H**). At the transcript (mRNA) level, PR gene expression was significantly reduced during the early phase of differentiation from induced pluripotent stem cells (iPSCs) to day 10 mammary embryoid bodies (mEBs) but were subsequently restored in day 20 organoids to levels comparable to those seen in undifferentiated iPSCs, revealing a biphasic (two-phase) expression pattern. Estrogen receptor alpha (ERα) protein was not detected by IHC in day 20 organoids (**Figure 24I**), consistent with the known downregulation (decrease) of ERα in actively proliferating cells during mammary gland development. At the transcript level, ERα displayed an early reduction paralleling that of PR, followed by a comparable recovery in mammary-like organoids to levels similar to those of undifferentiated iPSCs. In contrast, estrogen receptor beta (ERβ) expression was not reduced during differentiation; instead, it showed a gradual increase from mEBs to mature organoids (**Figure 25**).

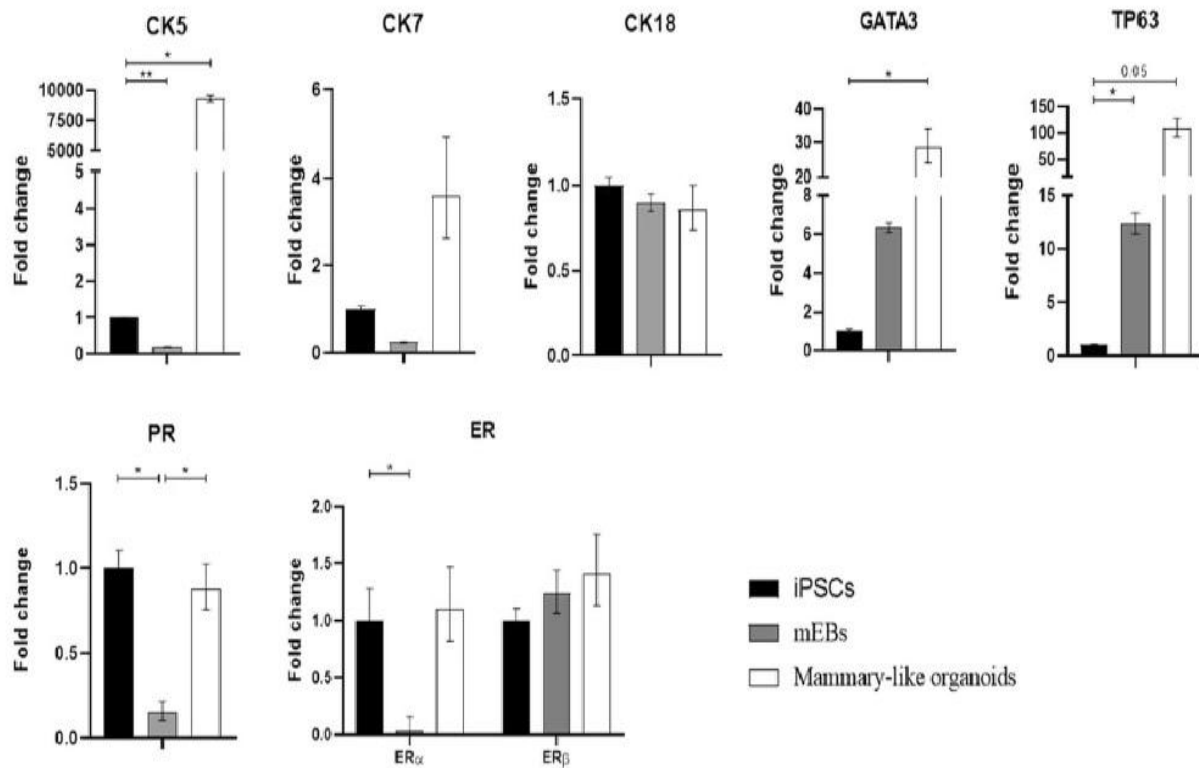


Figure 25: Gene expression of luminal markers (CK5/7 and GATA3), basal markers (CK18 and TP63), and hormone receptors (PR, ER α , and ER β) in iPSCs, 10-day mEBs, and 20-day mammary-like organoids. Histograms show fold change in gene expression; error bars indicate $\pm$ SEM.

4.6.3. Temporal Relationship Between Progesterone Receptor and GATA3

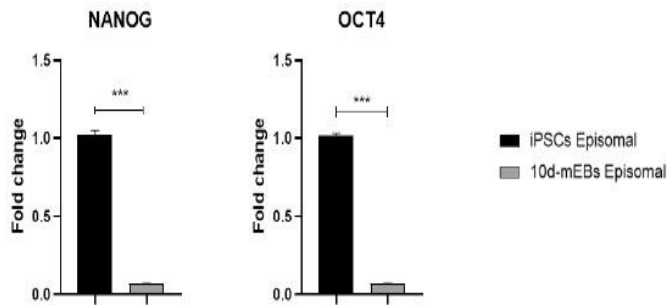
Undifferentiated iPSCs, which expressed elevated levels of PR, showed low GATA3 expression. During the transition to the day 10 mEBs stage, PR and GATA3 displayed an inverse temporal pattern: PR expression declined while GATA3 showed a trend of upregulation. By the day 20 organoid stage, GATA3 had reached significantly higher levels, and this increase occurred concomitantly with the restoration of PR expression. The simultaneous presence of both PR and GATA3 in mature mammary-like organoids is consistent with the expression pattern observed

during normal mammary gland development, where both factors contribute to luminal epithelial identity and hormone responsiveness.

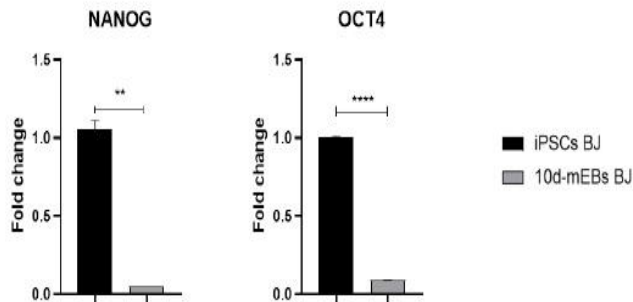
4.6.4. Loss of Pluripotency Markers

Consistent with the differentiation trajectory, the expression of the pluripotency-associated transcription factors NANOG and OCT4 decreased substantially from undifferentiated iPSCs to

A



B



the day 10 mEBs stage, confirming that the cells had existed in the pluripotent state and were progressing along the mammary differentiation programme (**Figure 26**).

Figure 26: Expression of the stemness markers **NANOG** and **OCT4** in (A) Episomal and (B) BJ cells. Histograms show fold change in gene expression; error bars indicate $\pm$ SEM.

Chapter 5

Discussion

The main objective of this thesis was to develop and evaluate a methodology for the generation of mammary-like organoids from human induced pluripotent stem cells (iPSCs), concurrently characterizing steroid hormone receptor expressions during the differentiation process. Chapter 4 elucidates that, starting from an episomal iPSC line, mammary embryoid bodies (mEBs) can be generated, which gradually attain non-neural ectodermal identity. When embedded in a three-dimensional Matrigel–collagen I matrix, these mEBs evolve into organoids that display the bilayered epithelial architecture typical of the native mammary gland. In parallel, a complementary multi-line investigation across four independently derived iPSC lines revealed that the progesterone receptor (PR) is constitutively expressed at the protein level in undifferentiated iPSCs—we found and confirmed this in the present work through extended differentiation and organoid generation. This chapter discusses the significance of these findings in the context of mammary gland biology, iPSC-based disease modelling, and the broader literature on steroid hormone signaling during development.

5.1. The Mammary Gland as a Developmental and Experimental Reference

The mammary gland is a complex, hormonally regulated organ whose development spans embryogenesis, puberty, pregnancy, lactation, and post-lactational involution. Its epithelium is organized into two principal populations—luminal and basal/myoepithelial—both of which are essential for ductal morphogenesis and secretory function. During embryogenesis, the rudimentary mammary bud forms through reciprocal epithelium–mesenchyme interactions mediated by

signaling pathways such as PTHrP/PTH1R and Wnt/ β -catenin, independently of sex steroids (Sreekumar et al., 2017; Ye et al., 2015). At puberty, however, the activation of estrogen and progesterone signaling drives branching morphogenesis, the formation of terminal end buds (TEBs), and the eventual generation of terminal ductal lobular units (TDLUs). During pregnancy, lactogenic hormones—principally progesterone and prolactin—promote lobuloalveolar differentiation and the maturation of secretory acini capable of milk production (Hennighausen & Robinson, 2005; Boss et al., 2018). After parturition, the abrupt decline in progesterone together with oxytocin-mediated stimulation triggers milk ejection, while sustained prolactin concentrations maintain lactation (Pang & Hartmann, 2007). The subsequent cessation of suckling initiates gland involution, characterized by epithelial cell apoptosis, lobular regression, and stromal remodeling mediated by caspases, matrix metalloproteinases, and macrophages.

Studying these processes *in vitro* has long been constrained by the limitations of two-dimensional monolayer cultures, which fail to recapitulate the three-dimensional organization and cell–cell interactions of the intact gland (Setia & Muotri, 2019). Three-dimensional models, including mammosphere and organoids, have addressed these limitations to varying degrees. Among these, mammosphere provide a valid system for studying tumor progression and recurrence, although they are genetically heterogeneous and difficult to obtain reproducibly (Al-Hajj et al., 2004; Clarke et al., 2006). Organoids offer a more complex and reproducible structure, capable of generating diverse cell types and recapitulating key stages of tissue development, whether starting from iPSCs or primary cells (Dontu et al., 2003; L. Zhang et al., 2017). Organoids derived from iPSCs are particularly attractive because they combine the scalability and genetic accessibility of pluripotent cells with the capacity to generate both luminal and basal lineages (Qu et al., 2017; Manganelli et al., 2024). The work described in this thesis builds directly on these foundations.

5.2. Mammary Embryoid Body Formation and Non-Neural Ectoderm Enrichment

The use of AggreWell™ plates enabled the generation of uniform sized spheroids, a prerequisite for minimizing experimental variability (Qu et al., 2017). Culture in MammoCult™ medium for ten days was designed to suppress the neural ectoderm subpopulation and enrich for non-neural ectoderm progenitors—the developmental stage that gives rise to basal and luminal mammary

lineages. qRT-PCR data obtained in this thesis confirmed this molecular trajectory: pluripotency markers OCT4 and SOX2 declined progressively from day 0 to day 10, indicating exit from the pluripotent state, while non-neural ectoderm and mammary commitment markers—P63, GATA3, AP2 α , and AP2 γ —showed concomitant upregulation. The early epithelial marker CK8 also increased during this interval, consistent with the acquisition of a luminal epithelial phenotype at the transcriptional level. Notably, PR transcript was also detectable at low levels throughout the ten-day mEBs culture period—an observation consistent with, and anticipating, the biphasic receptor expression pattern that is fully characterized in the longitudinal multi-line analysis described in section 5.4.

The expression pattern of GATA3 is particularly noteworthy: low expression was observed on the initial days (days 0 and 2), followed by a progressive increase reaching higher levels at day 10. This pattern suggests an enrichment of luminal lineage progenitors, recalling what occurs during puberty, when the rise in GATA3 is associated with the formation of TEBs—composed of alveolar and ductal progenitors—and regulates the formation of ductal branches (Fata et al., 2001; Hurtado et al., 2011; Kouros-Mehr et al., 2006). TP63, a marker expressed by cap cells and associated with the basal population, also showed an increase during differentiation, supporting the hypothesis of the presence of bipotent progenitors from which both luminal and basal cells derive. The upregulation of AP2 α is consistent with its established role in directing commitment toward the mammary lineage (Jäger et al., 2010; Sreekumar et al., 2017).

Despite the successful enrichment, the mEBs population remained heterogeneous at day 10. Although the neural ectoderm marker TUBB3 decreased during the first days of culture, PAX6 exhibited an upward trend at certain time points, and FOXG1 expression increased by day 10. These findings indicate that, despite successful enrichment of the non-neural programme, a residual neural progenitor population persists. This observation is consistent with the report by Qu et al. (2017), who noted the same limitation in their mEBs cultures, and underscores a well-recognized challenge in iPSC-based differentiation protocols: the difficulty of eliminating neural populations, which tend to emerge as a default fate during embryoid body formation. Future protocol refinements—such as the incorporation of neural fate inhibitors, modulation of the induction microenvironment, or adjustment of MammoCult™ treatment timing—could reduce this heterogeneity and improve population purity.

5.3. Constitutive Expression of Progesterone Receptor in iPSCs

One of the central and most unexpected findings of this thesis is the constitutive expression of PR protein in undifferentiated iPSCs. This result was first reported by our group and was confirmed across four independently derived iPSC lines generated from two distinct somatic cell types (CD34⁺ cord blood progenitors and skin fibroblasts) using four different reprogramming methods (episomal vectors, Sendai virus, retrovirus, and lentivirus). In all cases, immunofluorescence revealed strong, specifically nuclear PR staining in iPSC colonies, whereas ER α protein was consistently undetectable. Importantly, PR protein levels were undetectable or very low in the original somatic cells, i.e., the CD34⁺ hematopoietic stem/progenitor cells (HSPCs) and BJ fibroblasts, from which these iPSC lines were generated. Taken together, these data suggest that PR expression is a feature of the reprogrammed pluripotent state and not an inherited property of the somatic cell of origin, since a large fraction of iPSCs expressed PR protein at levels well above those of the corresponding parental populations (Manganelli et al., 2024).

At the transcript level, iPSCs showed low but detectable PR mRNA and markedly reduced ER α mRNA relative to the MCF7 positive control, whereas ER β was actively transcribed with variable, line-specific upregulation. ER α protein was undetectable in all iPSC lines tested. These observations suggest a dissociation between PR mRNA abundance and protein expression in iPSCs: despite low transcript levels, active translation and/or protein stabilization of PR protein occur—a phenomenon that may reflect post-transcriptional regulatory mechanisms or enhanced translational efficiency specific to the pluripotent state.

Constitutive expression of PR in iPSCs is consistent with previous findings in embryonic stem cells (ESCs). Hong et al. (2004) demonstrated the expression of ER α/β , glucocorticoid receptor, and PR genes in human ESCs and embryoid bodies. Hou and Gorski (1993) showed that there are distinct expressions of ER and PR genes during preimplantation development in mice up to the blastocyst stage. PR, and especially the PR-A isoform, has been demonstrated to be expressed in ESCs and to induce progesterone-dependent effects in early embryogenesis (Gallego et al., 2009, 2010). ER β has been involved in the maintenance of self-renewal and pluripotency (Loh et al., 2006; Ivanova et al., 2006; Carbognin et al., 2023). These discoveries are extended to iPSCs and suggest that reprogramming of somatic cells to a pluripotent state reactivates PR expression – possibly mimicking certain characteristics of the ESC-like hormone receptor programme. Thus,

PR expression could occur in early phases of reprogramming when cells gain ESC-like properties and could be related to developmental interaction between pluripotent and primordial germ cells (Ratajczak, 2017; Ratajczak et al., 2019).

These observations lead to some significant functional considerations. PR exists as two major isoforms, PR-A and PR-B, transcribed from different estrogen-responsive promoters. PR-A can serve as a dominant repressor of both PR-B and ER in a promoter and cell-specific manner (Vegeto et al., 1993; Wen et al., 1994). Progesterone has been shown to both drive (López-González et al., 2011) and inhibit (Kang et al., 2016) ESC differentiation to specific lineages and to influence the development and physiology of steroid-hormone-responsive tissues (Goletiani et al., 2007; Scarpin et al., 2009). Furthermore, the DNA-repair tumor suppressor protein, *BRCAl*, has been found to interact with and control both ER α and PR transcriptional activity (Fan et al., 1999, 2001; Ma et al., 2006). Together, these data suggest that regardless of the cell of origin or reprogramming technology used, PR may have a functional role in cell reprogramming to iPSCs and, because PR is already expressed in the undifferentiated state, it cannot be used as a specific marker of iPSC-based differentiation to mammary or other hormone-responsive lineages.

5.4. Dynamic Regulation of PR During Mammary Organoid Differentiation

The longitudinal profiling of PR expressions across the differentiation trajectory from iPSCs through mEBs to mammary-like organoids revealed a biphasic pattern. PR gene expression was significantly reduced during the early phase of differentiation from iPSCs to day-10 mEBs but was subsequently restored in day-20 organoids to levels comparable to those of undifferentiated iPSCs. ER α showed an early decline paralleling that of PR, followed by a similar recovery in mammary-like organoids to levels comparable with undifferentiated iPSCs, whereas ER β exhibited a trend of progressive upregulation across the differentiation time course (Manganelli et al., 2024).

These expression dynamics are consistent with the known downregulation of ER α in actively proliferating cells during mammary gland development (Bocchinfuso & Korach, 1997; Zeps et al., 1998; Mallepell et al., 2006; Clarke et al., 1997).

A particularly informative feature of this trajectory was the temporal interplay between PR and GATA3. Undifferentiated iPSCs, which expressed elevated levels of PR, showed low GATA3 expression. During the transition to day-10 mEBs, PR and GATA3 displayed an inverse pattern: PR declined while GATA3 showed a trend of upregulation. By day-20 organoid stage, GATA3 was expressed at much higher levels, coinciding with the restoration of PR expression. This pattern fits with the well-established functional relationship between PR and GATA3 during mammary gland development; PR activation downregulates GATA3 by transcriptional repression (Izzo et al., 2014), but both factors are ultimately co-expressed in mature luminal cells and have a role in luminal epithelial identity and hormone responsiveness (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). This conclusion was further reinforced by the modulation of PR downstream target genes. CK5, a progesterone-inducible gene (Axlund et al., 2013), was expressed at extremely low levels in iPSCs and day-10 mEBs but was significantly elevated in day-20 organoids.

Immunohistochemical analysis of day-20 organoids revealed strong nuclear PR staining in luminal epithelial cells consistent with the transcript results and indicative of a mature, hormone-responsive luminal cell population. The simultaneous absence of detectable ER α protein at this time is consistent with the negative correlation between ER α expression and active cell proliferation documented during mammary gland development (Petersen et al., 1987; Shyamala et al., 2002).

5.5. Validation of Mammary Epithelial Identity in Organoids

Immunohistochemical characterization of the organoids generated in this thesis confirmed the presence of both luminal and basal/myoepithelial compartments, recapitulating the bilayered architecture of the native mammary gland. Strong peripheral staining for CK5/6 and TP63 around ductal and alveolar structures indicated the presence of an active basal and myoepithelial compartment: CK5/6 is expressed in basal and myoepithelial cells, while TP63 is a key marker of basal progenitors essential for maintenance of the stem cell population and basal layer formation. CK14 staining further confirmed the presence of differentiated basal cells. As a high-molecular-weight cytokeratin expressed by basal and myoepithelial cells, CK14 is frequently used as a marker for stem or progenitor cells of this lineage (Arendt et al., 2014). Its presence in the organoids suggests that the protocol maintained the capacity to generate and stabilize the basal compartment.

In the luminal compartment, GATA3 positivity—with nuclear staining evident in cells lining the alveoli and ducts—demonstrated correct luminal differentiation. CK18 positivity confirmed the presence of differentiated luminal cells capable of developing functional internal epithelial structures. α -Smooth muscle actin (SMA) staining confirmed myoepithelial cell differentiation forming the outer basal layer surrounding the ducts and alveoli, providing structural support and contributing to the contraction necessary for milk ejection. The localization of SMA-positive cells around the luminal structures demonstrated that the organoids maintained the characteristic bilayered architecture of the mammary gland. These findings agree with the results reported by Qu et al. (2017), who described TP63 and CK14 as indicators of proper basal population formation in iPSC-derived organoids, and consistent with findings from our group, who reported GATA3 and CK18 as markers of correct luminal maturation.

Organoids generated from the Episomal iPSC line were further cultured to day 35, during which progressive branching morphogenesis was observed: cellular extensions emerged from the central body of each organoid and extended into the surrounding Matrigel matrix, forming branched structures consistent with the development of a rudimentary ductal tree. This structural maturation was supported by a concurrent molecular signature of lactogenic competence. PRLR expression was progressively upregulated during dome culture, reaching substantially higher levels by day 20 compared with the near-baseline levels detected on day 5. RT-PCR analysis confirmed distinct PRLR expression in end-stage organoids, whereas only a faint signal was detectable in day-10 mEBs. PR expression was also confirmed at the transcript level in mature organoids, collectively indicating that the differentiated structures had acquired a hormone-responsive luminal epithelial phenotype. The progressive acquisition of PRLR expression provides compelling molecular evidence for the organoids' capacity to respond to lactogenic stimuli, and supports the feasibility of the lactation induction experiments, as outlined in section 5.7. The matrix, composed of Matrigel and type I Collagen, was designed to mimic stroma–epithelium interactions and to support lateral branch organization, as matrix density is known to play a crucial role in epithelial differentiation and the fate of stem progenitors (Butcher et al., 2009; Fata et al., 2004; Guilak et al., 2009; Maller et al., 2010). These results are consistent with reports by Qu et al. (2017) demonstrating that iPSC-derived organoids can be induced toward mammary secretory function, and with Lee et al. (2023), who showed that human three-dimensional models can recapitulate key aspects of mammary tissue, including secretory properties.

5.6. Implications for iPSC-Based Disease Modelling

The constitutive production of PR in undifferentiated iPSCs carries significant implications for the design and interpretation of iPSC-based disease models, particularly those involving PR-responsive organs such as the breast and ovaries. PR is already present in the undifferentiated state and cannot be used as a specific differentiation marker, and the baseline expression of PR needs to be considered when designing and evaluating research involving steroid hormone signaling in iPSC-derived mice. This is especially important in breast cancer models, where the hormone receptor status of cells is a key predictor of tumor biology and therapy response.

These findings also overlap with the wider understanding of hormone receptor expression in stem and progenitor cell groups. ER α was extensively expressed in murine HSCs and enabled estrogen-driven self-renewal (Nakada et al., 2014), and functional sex hormone receptors were found in murine and human HSPCs (Mierzejewska et al., 2015; Abdelbaset-Ismail et al., 2016). We found that neither ER α nor PR were discovered at meaningful quantities in CD34 + HSPCs or BJ fibroblasts, differentiated somatic cells lacking pluripotency programme. The absence of PR in these somatic populations and its presence after reprogramming to the pluripotent state support the idea that PR expression is linked to the stem cell-like program reactivated during iPSC generation, perhaps reflecting a developmental relationship with primordial germ cells that express sex hormone receptors (Ratajczak, 2017; Ratajczak et al., 2019).

5.7. Future Perspectives

The iPSC-based mammary organoid platform established in this thesis provides a foundation for broad, clinically relevant application and opens several ways of future investigation. A primary objective will be the integration of CRISPR-based gene editing tools to introduce or correct *BRCA1/2* mutations in patient-specific iPSC lines, enabling direct modelling of breast cancer initiation and the mechanistic dissection of genetic variants of uncertain significance (VUS) within a controlled, physiologically relevant environment. In parallel, the demonstrated capacity of these organoids to express milk proteins — including LALBA and CSN2 — opens the way toward fully mature lactational models, which could serve as powerful tools for drug screening, regenerative

medicine applications, and personalized oncology approaches targeting hormone-responsive breast pathologies.

5.8. Conclusions

The thesis demonstrates a reproducible and physiologically relevant protocol for generating three-dimensional mammary-like organoids from human iPSCs, capable of recapitulating the bilayer architecture of native mammary tissue, with coherent expression of luminal markers - GATA3, CK18, CK7, and basal/myoepithelial markers - CK5/6, CK14, TP63, and SMA. The progressive acquisition of PRLR expression and transcriptional evidence of milk protein genes also suggest functional maturation toward a lactational phenotype, considerably increasing the biological significance of the model.

In parallel, the discovery of constitutive expression of progesterone receptor protein in four independently derived iPSC lines, irrespective of somatic cell origin and reprogramming method, identifies PR as an intrinsic attribute of the reprogrammed pluripotent state. Its dynamic regulation during differentiation mirrors key aspects of normal mammary gland development, underscoring the developmental fidelity of the system. Together, these findings define a robust iPSC-based mammary platform that not only advances our understanding of progesterone signaling in pluripotent and differentiating cells, but also opens new opportunities for disease modelling, functional genomics, and precision studies of hormone-responsive breast pathologies.

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Appendix

Stem Cell Reviews and Reports
<https://doi.org/10.1007/s12015-024-10776-6>



Progesterone receptor is constitutively expressed in induced Pluripotent Stem Cells (iPSCs)

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Accepted: 7 August 2024
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Abstract

Induced Pluripotent Stem Cells (iPSCs) are nowadays a common starting point for wide-ranging applications including 3D disease modeling (i.e. organoids) and in future regenerative medicine. Physiological processes like homeostasis, cell differentiation, development and reproduction are tightly regulated by hormones through binding to their transmembrane or nuclear receptors of target cells. Considering their pleiotropic effect, take into account also their expression in an iPSCs-based disease modeling would better recapitulate the molecular events leading to 3D organoid development and disease study. Here we reported the expression pattern of estrogen receptor (ER α) and progesterone receptor (PR) in four different iPSCs, obtained from CD34+ progenitor cells and skin fibroblasts with four different methods. Expression of ER α and PR mRNA were significantly downregulated in iPSCs as well as fibroblasts compared to MCF7 positive control. Immunofluorescence (IF) staining detected only the expression of PR protein in all the different iPSCs cell lines, while ER α was not detectable. By flow cytometry analysis we observed that the ~65% of the total population of iPSCs cells expressed only PR, with 100% fold increase compared to HSPCs and fibroblasts, while ER α was not expressed. Our results collectively demonstrated for the first time that the reprogramming of somatic cells into iPSCs leads to the expression of PR receptor.

Keywords CD34 · Fibroblasts · Induced pluripotent stem cells (iPSCs) · Progesterone receptor · Estrogen receptor · Differentiation

