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Unraveling the expression and regulation of intercellular junctions in normal and Brominated Flame Retardants (BFRs) treated murine mammary glands

By

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Dedication

This journey was one of the toughest and the most enriching paths I have ever walked across my life and it wouldn't be a triumph without the presence and support of many people. Among them there are few ones which their support and presence during these years is unforgettable. I would take this chance and dedicate this thesis

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For his understanding, support, and self-devotion

I would always remember that your great spirit made this goal achievable. Thank you for prioritizing my success over your needs

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For sending those positive vibes and heart-warming hopes

For their loving characters and those endless encouragements despite of being six thousand miles apart

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Foreword

This thesis is written based on INRS Guide-2016, article based version in order to fulfill the last requirement of this program, Doctorate in biology, from INRS-Institut Armand Frappier.

It consists of abstract (English and French versions), synopsis, introduction, publications, conclusion and perspective, annex and bibliography which will be further discussed in below.

Based on INRS rules, a synopsis is required when a thesis or dissertation is written in English. This section includes the review of literature and hypotheses rising form it, research objectives, methodology, and results in much more detail than the abstract does and refer to tables and figures in the main text.

Chapter 1, introduction, substantially consists of the review of literature addressing our main hypothesis. The figures used are either from literature or provided in our laboratory in order to better explain the pointed facts and haven't been used in the publications. This chapter ends with the hypothesis and objectives of the research performed during the PhD program.

Chapter 2, publications, includes the core of our findings during this program starting from September 2012 till April 2017. It is comprised of three scientific papers, two published and one submitted which is currently under review. Each individual paper is provided in full text format including the figures and references.

Chapter 3, conclusion and perspective, relates the findings and summarizes the contribution of our findings to the progress of science while addressing the several future approaches. It consists of few schemes in order to help visualizing the main findings.

Chapter 4, annexe, contains a scientific paper for which I am co-author. This paper which is almost ready to be submitted, aims to determine the effects of a perinatal exposure to the same mixture of Brominated Flame Retardants that has been used on dams (Chapter 2 of the thesis, publication 3) on mammary gland development in pups. My contributions to this paper include, animal sacrifice, tissue harvesting, whole mount experiments, study design and techniques setup, while most of the other experiments have been conducted by a master student from our laboratory.

Finally chapter 5, contains the bibliography used in this thesis. It should be noted that the references used for each scientific papers in chapter 2 are provided at the end of each article and are not included in chapter 5.

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List of acronyms and abbreviations

Adherens Junctions = AJs

Adult (week 10) = W10

Androgen receptor = AR

Before puberty (week 4) = W4

Brominated Flame Retardants = BFRs

Connexins = Cxs

Epidermal growth factor receptor = EGFR

Embryonic day … = i.e. E11, E18

Endocrine disruptors = EDs

Epidermal growth factor = EGF

Epithelial mesenchymal transition= EMT

Estrogen = E2

Estrogen receptor = ER

Extracellular matrix = ECM

Fibroblast growth factor receptor= FGFR

Fibroblast growth factor = FGF

Gap junctions = GJs

Glucocorticoid receptor = GR

Growth hormone = GH

Insulin-growth factor 1= IGF-1

Involution day 1 = In1

Involution day 8 = In8

Lactation day 14 = L14

Lactation day 7 = L7

Matrix metalloproteinase = MMP

Placental lactogen = PL

Pregnancy day 18 = P18

Pregnancy day 8 = P8

Progesterone = P4

Progesterone receptor = PR

Prolactin = PRL

Prolactin receptor = PRLR

Puberty (week 6) = W6

Receptor activator of nuclear factor kappa-B ligand= RANKL

Terminal end buds = TEBs

Thyroid hormone receptor = TR

Tight junctions = TJs

Abstract

Mammary glands undergo diverse structural changes across the mammals' life span. Cell-cell interactions by means of Gap (GJs), Tight (TJs) and Adherens junctions (AJs) providing cross-talk between epithelial cells, are required for proper function and development of the gland. It has been shown that junctional proteins interplay regulates each other's function in other tissues. Mammary glands' development is also tightly regulated by hormones. Because junctional proteins expression is also influenced by hormones, exposure to some endocrine disruptors (EDs) was reported to abrogate intercellular junctions. The objectives of this research were to: 1) assess the expression profile, localization as well as interaction of GJs, TJs and AJs across post-natal mammary gland development, and 2) evaluate the effect of brominated flames retardants (BFRs) on intercellular junctions.

For the first objective mammary glands from mice were sampled at various developmental stages. RT-qPCR and western-blot demonstrated differential expression pattern for all members of gap, tight and adherence junctions tested across development. Immunofluorescence showed that junctional proteins were localized at the cell-cell interface suggesting the existence of a junctional nexus. Co-immunoprecipitation established that junctional protein interplay together and form nexuses which are specific to different stages of development. Our results established that junctional interactions vary throughout development, likely following hormonal changes, and suggest that these interactions might regulate mammary gland function and stability of the nexus at cell membrane.

For the second objective, female rats received different doses of a BFRs mixture prior to mating until weaning day when mammary glands were collected. Western blot showed that BFRs had no adverse effect in milk synthesis, hormonal receptors and intercellular junctions. While BFRs did not change β-catenin levels, it resulted in a significant down-regulation in phospho-ser675 β-catenin (p-β-cat^{Ser675}) at low dose, likely in a PKA-dependent manner. Co-Immunofluorescence assay showed that p-β-cat^{Ser675} is localized at cell membrane and confirm the decreased levels at low and medium doses. Finally, our results established that the interaction between p-β-cat^{Ser675} and E-cadherin is reduced in low dose treated animals. These results suggested that while BFRs exposure did not result in major functional issues, it did affect the role of p-β-catenin^{ser675} in cell adhesion.

Together, our results demonstrated that junctional nexus are in constant remodeling during mammary gland development, likely reflecting the needs for cell-cell interactions. Moreover, EDs can

impact these interactions by affecting cell signaling. More studies are required to fully understand the long-term impacts of these dysregulations.

Keywords; Mammary gland, Murine, Development, Brominated Flame Retardants (BFRs), Junctions, interaction, Connexins (Cxs), Cadherins, β-catenin, p-β-cat^{Ser675}

Résumé

La structure des glands mammaires est en constant remodelage et nécessite une régulation hormonale précise. Les jonctions communicantes, serrées et adhérentes sont nécessaires pour le bon fonctionnement et développement des glandes mammaires. Il a été démontré que les protéines jonctionnelles interagissent et que leur expression est régulée par des mécanismes communs dans d'autres tissus, dont par des voies activées par les hormones. Certains perturbateurs endocriniens (EDs) peuvent également modifier l'expression des protéines jonctionnelles. Notre hypothèse de recherche est donc que l'expression des protéines jonctionnelles et leurs interactions varient selon le stade de développement des glandes mammaires, et qu'une exposition à des EDs pourrait influencer ces interactions. Ce projet compte deux objectives : 1) déterminer les profils d'expression, la localisation et les interactions de composants des jonctions intercellulaire à différents stades de développement des glandes mammaires, et 2) évaluer l'effet d'un mélange de retardateurs de flamme Bromée (BFR) sur les jonctions intercellulaires.

Pour le premier objectif, des glands mammaires de souris ont été collectés à différents stades de développement. Nos résultats ont démontré que l'expression des protéines des jonctions communicantes, serrées et adhérentes étudiées est modifiée durant le développement et qu'elles sont présentes à l'interface entre deux cellules. De façon intéressante, nous avons démontré que les interactions entre les différentes protéines jonctionnelles diffèrent durant le développement. L'ensemble de nos résultats suggère que l'expression et les interactions entre les protéines jonctionnelles pourraient être régulées par les changements hormonaux, reflétant des besoins fonctionnels différents.

Pour le deuxième objectif, des rats femelles ont été exposés par la diète à un mélange de BFRs avant l'accouplement jusqu'à la fin de l'allaitement, et sacrifiées au jour du sevrage. Aucune différence n'a été observée dans plusieurs protéines étudiées. Bien que les niveaux totaux de β-caténine furent inchangés, l'exposition à la dose la plus faible de BFRs a réduit la forme phosphorylée Ser675 de β-caténine (p-β-cat^{Ser675}), probablement via une baisse d'activité de PKA. De plus, le traitement a diminué l'interaction entre les p-β-cat^{Ser675} et E-cadhérine. Ces résultats démontrent qu'une exposition à un mélange de BFRs affecte peu la fonction des glandes mammaires, mais peut inhiber le rôle de p-β-cat^{ser675} dans complexe d'adhésion cellulaire.

L'ensemble de nos résultats démontrent que le nexus jonctionnel est en constant remodelage durant le développement des glandes mammaires, probablement en fonction des besoins fonctionnels. De plus, des EDs peuvent modifier ces interactions. Les conséquences à long terme de ces perturbations restent à déterminer.

Des mots clés; glands mammaires, murin, développement, Retardateurs de Flamme Bromée (RBF), jonctions, interaction, connexines, cadhérine, β-caténine, p-β-cat^{Ser675}

Synopsis (FR)

Ce synopsis est un résumé succinct de la thèse rédigée en anglais. Il passe brièvement en revue les différentes sections présentées dans le document complet. Il est composé de quatre parties; 1) une revue de la littérature, 2) l'hypothèse de recherche et les objectifs, 3) une section décrivant le matériel, les méthodes et résultats, et 4) une discussion et des conclusions en lien avec les résultats obtenus.

Revue de la littérature

Le Développement et la structure des glandes mammaires

Les glandes mammaires sont des organes spécialisés qui jouent un rôle nutritionnel chez toutes les espèces de mammifères. Elles sont constituées d'un épithélium bicouche et d'un stroma comprenant principalement des fibroblastes, des cellules adipeuses, des cellules du système immunitaire et une matrice extracellulaire. Les humains possèdent une seule paire de glandes mammaires, tandis que les souris en ont cinq et les rats six. Bien qu'il y ait quelques différences dans la structure des glandes mammaires entre les humains et les rongeurs, principalement dans la composition du stroma, le développement des glandes mammaires est très similaire entre ces espèces.

Le développement des glandes mammaires commence avant la naissance, mais se déroule majoritairement après la puberté en réponse aux changements hormonaux (Richert, Schwertfeger et al. 2000). Lors du développement embryonnaire, un canal galactophore primaire avec quelques branches se forme, entouré de mésenchyme et de stroma. Le mamelon est également formé à la surface de la peau. Ce canal primordial demeurera dormant jusqu'à la puberté (Figure 1).

Au moment de la puberté, l'augmentation des niveaux d'hormones circulantes, principalement des estrogènes, induira une prolifération soutenue des cellules épithéliales qui formeront alors une structure appelée bourgeons terminaux (Terminal end buds, TEBs) (Neville, McFadden et al. 2002). Les TEBs sont composés des cellules cubiques basales entourant les cellules apoptotiques responsables de produire la cavité du canal (Figure 2) (Daniel CW 1987). Ces sites de prolifération intense permettront l'élongation des canaux et leur ramification. Des branches tertiaires seront également formées, principalement sous l'action de la progestérone (Atwood, Hovey et al. 2000). Il y aura également augmentation du volume des glandes mammaires et des tissus graisseux. Chez l'adulte, la glande mammaire sera composée d'un épithélium ramifié contenu dans un stroma (Figure 3). À chaque cycle menstruel, les glandes subiront

des cycles de prolifération et de régression, suivant principalement les variations d'estrogènes et de progestérone (Richert, Schwertfeger et al. 2000) (Sternlicht 2006).

Une seconde phase de prolifération et de différentiation se déroulera lors de la grossesse (Oakes, Hilton et al. 2006) (Daniel CW 1987). Chez la femelle gestante, les canaux se ramifieront de nouveau afin de former les alvéoles, les structures responsables de la sécrétion du lait, et la quantité du tissue adipeux diminuera progressivement (Richert, Schwertfeger et al. 2000, Sternlicht 2006). L'épithélium sera alors composé de canaux lactophores, de canaux intra-lobulaires, de lobules et d'acini (Figure 2 & 3). Cette seconde phase de différentiation et prolifération permettra l'obtention d'un organe fonctionnel pour sécréter le lait au cours de la lactation (Neville, McFadden et al. 2002). Finalement, après la période de lactation, lors d'une phase de régression appelée involution, l'accumulation de lait et l'absence de stimuli induiront l'apoptose des cellules épithéliales et un remodelage du stroma et des tissus adipeux afin de retourner à l'état «pré-grossesse» (Neville, McFadden et al. 2002).

L'épithélium des glandes mammaires est constitué de deux couches de cellules : les cellules luminales entourées d'une couche de cellules basales, composée majoritairement de cellules myoépithéliales (Figure 4). L'épithélium comporte également des populations de cellules souches et progénitrices permettant la régénération des tissus lors des cycles menstruels et lors de la grossesse (Visvader 2009) (Inman, Robertson et al. 2015). Finalement, les cellules épithéliales qui entourent des acini sont extrêmement polarisées afin de conserver la compartimentation entre l'épithélium et la lumière. Les interactions entre les cellules luminales et les cellules myoépithéliales, ainsi qu'avec le stroma, sont nécessaires afin de permettre cette polarité et à la formation d'un épithélium fonctionnel.

Les jonctions intercellulaires dans les glandes mammaires

Les interactions cellulaires sont nécessaires pour le bon fonctionnement et le développement des tissues comme les glandes mammaires. Trois types des jonctions cellulaires participent à ces interactions : les jonctions lacunaires (mieux connues sous les appellations « jonctions gaps » ou jonctions communicantes), les jonctions serrées et les jonctions adhérentes (Figure 5). Ces jonctions cellulaires forment une structure dynamique qui se crée, se remodèle et se dissocie au cours du développement et selon les besoins du tissu.

Les jonctions gap/communicantes

Les jonctions gap (GJs) sont des canaux qui interconnectent directement les cytoplasmes de deux cellules voisines et permettent du passage de petites molécules (taille<1.2 kD) entre les cellules (Stewart, Simek and Laird, 2015) (Locke and Harris 2009). Elles jouent un rôle dans la croissance, le développement embryonnaire, la différentiation des tissus et les fonctions tissulaires. Leur dérégulation a été associée à plusieurs maladies, dont au cancer (Naus and Laird, 2010). Elles sont constituées par une famille de protéines appelées connexines (Cxs) (El-Sabban, Abi-Mosleh et al. 2003) (Warner 1992). Les Cxs sont des protéines transmembranaires en forme de « M » constituées de deux loupes extracellulaires (E1 et E2), de quatre domaines transmembranaires (M1-M4), d'une loupe cytoplasmique, d'une queue amino-terminal (NT) et d'une queue carboxyl-terminal (CT) (Dbouk, Mroue et al. 2009). Les séquences des Cxs sont hautement conservées entre les membres de la famille et entre les espèces. Les principales divergences se trouvent dans la loupe cytoplasmique et la CT, et sont responsables des différences dans la sélectivité des canaux formés par les différentes Cxs (El-Sabban, Abi-Mosleh et al. 2003) (Figure 6). Six Cxs s'assemblent dans une structure hexamérique, formant ainsi un demi-canal appelé connexon. L'assemblage de deux connexons provenant de cellules adjacentes constitue un canal jonctionnel. Les canaux jonctionnels se regroupent dans des zones spécifiques de la membrane plasmique appelées plaques jonctionnelles. Chaque plaque peut contenir entre 100 jusqu'à 1000 canaux. Les connexons peuvent être constitués d'un seul type de Cxs (homomérique) ou de différentes Cxs (hétéromérique). L'attachement de deux connexons de composition identique résultera en la formation d'un canal homotypique, alors que deux connexons de composition différente formeront un canal hétérotypique (El-Sabban, Abi-Mosleh et al. 2003) (Figure 6).

Il existe 21 connexines en tout chez l'humain, dont quatre sont exprimées dans les glandes mammaires de la souris, soit Cx43, Cx26, Cx30 et Cx32, et deux chez l'humain, Cx26 et Cx43 (Oyamada, Oyamada et al. 2005) (Sohl and Willecke 2003)(McLachlan, Shao et al. 2007) (Figure 7). Des études utilisant des souris knockout (KO) ou transgéniques ont permis de mieux comprendre le rôle des Cxs dans le développement et le fonctionnement des glandes mammaires. Les souris KO pour Cx30 et Cx32 sont fertiles et aucun problème de lactation n'a été rapporté (Teubner, Michel et al. 2003) (Nelles, Butzler et al. 1996). Les souris KO pour Cx26 et Cx43 meurent *in utero* ou peu de temps après la naissance, empêchant ainsi l'étude du développement des glandes mammaires dans ces modèles (Gabriel, Jung et al. 1998) (Reaume, de Sousa et al. 1995). Par contre, des modèles de KO conditionnel, spécifique aux glandes mammaires, ont démontré qu'une inhibition de Cx26 avant la puberté empêche la formation lobulo-alvéolaire, tandis que la diminution de Cx26 pendant la gestation et la lactation n'a aucun effet (Stewart, Plante et al. 2014) (Bry, Maass et al. 2004). Des souris mutantes, appelées *Gja1*$^{Jrt/+}$

(ou G60S), ont une mutation dominante-négative dans le gène codant pour la Cx43, résultant en une baisse d'environ 70-90% des niveaux de Cx43 dans les différents tissus (Flenniken, Osborne et al. 2005). Chez ces souris, il a été démontré que la baisse de Cx43 résulte en un délai dans la formation de la glande mammaire et empêche la lactation (Plante and Laird 2008).

Les jonctions serrées (TJs)

Les jonctions serrées (TJs) permettent le rapprochement des membranes de deux cellules adjacentes afin de former une barrière semi-perméable (Fromter 1972) (Chalcroft and Bullivant 1970). Elles sont ainsi impliquées dans la polarité en permettant le passage sélectif de molécules de part à d'autre de la barrière. Elles sont nécessaires à l'homéostasie et la perméabilité des tissus glandulaires, tel que les glandes mammaires (Tsukita, Furuse et al. 2001) (Brennan, Offiah et al. 2010). Les composants des jonctions serrées sont classifiées en 1) protéines intégrales membranaires, telles que l'occludine et les claudines, 2) protéines cytoplasmiques, comme la famille des zona occludens (ZO), et 3) les protéines de signalisation (Itoh and Bissell 2003) (Figure 8). Dans la glande mammaire, les TJs sont essentielles pour empêcher les fuites du lait à travers les cellules épithéliales. Leur fonction est régulée principalement par les hormones lactogènes (la prolactine et les glucocorticoïdes) et la progestérone (Nguyen, Parlow et al. 2001) (Stelwagen, McFadden et al. 1999) (Rubenstein, Guan et al. 2003).

Les jonctions adhérentes (AJs)

Les AJs sont présentes dans tous les épithéliums et sont impliquées dans l'attachement de cellules voisines et aussi entre l'épithélium et la membrane basale. Elles sont nécessaires à la maturation des tissus, au développement et à la morphogenèse, et sont perturbées lors de la transition épithélio-mésenchymateuse (Conacci-Sorrell, Zhurinsky et al. 2002). Ces jonctions sont majoritairement constituées des cadhérines et des caténines. Les cadhérines sont des protéines d'adhésion transmembranaires, qui ont été nommées d'après les tissus à partir desquels elles ont été identifiées pour la première fois : E-cadhérine (cellules épithéliales), N-cadhérine (système nerveux), VE-cadhérine (endothélium vasculaire) et P-cadhérine (Placenta) (Nose and Takeichi 1986) (Rudini and Dejana 2008). Les cadhérines sont constituées de cinq domaines extracellulaires (EC) liés au calcium et d'une queue cytoplasmique hautement conservée, incluant le site de la liaison pour β-caténine et/ou γ-caténine (Figure 9) (Saito, Tucker et al. 2012). Les domaines EC des cadhérines de cellules adjacentes se lient, formant ainsi un brin dimérique. Les caténines permettent l'attachement du complexe au cytosquelette d'actine.

En plus de son rôle au niveau des AJs, β-caténine peut également agir comme facteur de transcription dans la voie de signalisation Wnt, par sa liaison au facteur de transcription TCF/LEF (Incassati, Chandramouli et al. 2010). Lorsque la β-caténine est libre dans le cytosol, i.e. non-liée à E-cadhérine, elle est généralement dégradée par le protéasome suite à une série de phosphorylation-ubiquitination (Incassati, Chandramouli et al. 2010). Par contre, suite à l'activation de la voie Wnt, β-caténine s'accumule dans le cytosol et peut transloquer vers le noyau pour agir sur des gènes cibles. Un équilibre entre ces deux fonctions est nécessaire au maintien de l'homéostasie du tissu et à la fonction des glandes mammaires; un dysfonctionnement a été associé au cancer du sein et à des problèmes développementaux dans la glande mammaire (Andrews, Kim et al. 2012) (Perez-Moreno and Fuchs 2006) (Rudini and Dejana 2008).

Les jonctions intercellulaires forment un complexe jonctionnel dans différents tissus

Les interactions cellulaires jouent des rôles essentiels dans l'intégrité des tissus et permettent la compartimentation des différentes composantes des glandes mammaires (Liu, Radisky et al. 2005) (Talhouk, Mroue et al. 2008). Il a été démontré que les protéines jonctionnelles interagissent ensemble et forment un nexus entre les cellules adjacentes dans différents tissus. Les interactions entre les Cxs et les protéines du cytosquelette, des TJs et des AJs sont nécessaires pour la stabilité, l'assemblage, le transport et la fonction des GJs dans les différents tissus (Herve, Bourmeyster et al. 2007) (Herve, Bourmeyster et al. 2004) (Kojima, Kokai et al. 2001) (Dbouk, Mroue et al. 2009). Cependant, le rôle et la régulation du nexus jonctionnel lors du développement des glandes mammaires sont très peu connus.

Les hormones et facteurs de croissance impliqués dans le développement des glandes mammaires

Les études ont démontrées que la croissance embryonnaire des glandes mammaires n'est pas sous contrôle hormonal (Sternlicht, Kouros-Mehr et al. 2006). Contrairement à la plupart des autres organes, la majeure partie du développement des glandes mammaires se déroule de façon post-natale, à partir de la puberté. Les hormones ovariennes et hypophysaires, ainsi que différents facteurs de croissance, jouent des rôles clés dans le développement du tissu mammaire (Figure 10&11) (Gumbiner 1996) (Haslam and Shyamala 1981). Elles sont également impliquées dans la régulation de certaines protéines jonctionnelles.

Les estrogènes; Les estrogènes sont des hormones stéroïdiennes essentielles pour la fonction du système reproducteur femelle. Au niveau de la glande mammaire, les estrogènes sont nécessaires à l'élongation des canaux galactophores, mais sont également impliqués dans la régulation de la sécrétion de la prolactine, des hormones FSH et LH de l'axe hypothalamus-hypophysaire et de la progestérone (Brisken and Ataca 2015) (Scully, Gleiberman et al. 1997) (Ruan, Monaco et al. 2005) (Hewitt and Korach 2000). Il existe deux types de récepteurs aux estrogènes : les récepteurs alpha et beta (ERα et Erβ). L'ERα est nécessaire pour le développement de l'épithélium mammaire, alors que le rôle d'ERβ n'est pas totalement compris (Mallepell, Krust et al. 2006).

La progestérone; La progestérone est une hormone stéroïdienne sécrétée par les ovaires sous l'action de la LH. Elle agit comme précurseur pour la synthèse des androgènes et des estrogènes, et inhibe l'expression du récepteur à la prolactine. Il existe également deux types des récepteurs pour la progestérone, PRA et PRB, qui possèdent des fonctions différentes dans les glandes mammaires. PRA est exprimé chez la souris vierge, tandis que PRB est particulièrement exprimé durant gestation. PRB est nécessaire pour le développement des branches tertiaires (tubuloalvéolaires) des glandes mammaires (Haslam and Shyamala 1981, Haslam 1989).

La prolactine; L'action combinée de la prolactine, de la progestérone et de la lactogène placentaire est nécessaire pour la différentiation des alvéoles et pour induire l'expression génique des protéines du lait (Brisken, Kaur et al. 1999) (Humphreys, Lydon et al. 1997, Brisken, Park et al. 1998). Le récepteur à la prolactine est exprimé dans les cellules épithéliales et est requis pour l'alvéologenèse et la sécrétion du lait.

Les hormones thyroïdiennes; Les hormones thyroïdiennes ne sont pas nécessaires à la croissance des canaux, mais sont impliqués dans le développement des alvéoles (Borellini and Oka 1989) (Brisken and Ataca 2015). Via leurs récepteurs, elles agissent en synergie avec la prolactine lors de l'alvéologenèse (Vonderhaar and Greco 1979).

Les facteurs de croissance; Plusieurs facteurs de croissance sont impliqués dans la prolifération et la différentiation cellulaire. **Le facteur de croissance ressemblant à l'insuline 1 (insulin-like growth factor I, IGF-1)** et **l'hormone de croissance (GH)** sont nécessaires pour la formation des canaux de la glande mammaire durant la période postnatale. IGF-I, en combinaison avec les estrogènes, stimulent également les bourgeons terminaux et alvéolaires. L'action synergique de la progestérone et de l'IGF-I permet la croissance de canaux galactophores et leurs ramifications (Sternlicht, Kouros-Mehr et al. 2006) (Richards,

Klotz et al. 2004). **Le facteur de croissance épidermique (epidermal growth factor, EGF)** agit en aval du récepteur de l'estrogène pour induire la croissance et la ramification des canaux (Sternlicht, Kouros-Mehr et al. 2006, Ciarloni, Mallepell et al. 2007).

En résumé, le développement de la glande mammaire est un processus comportant des phases de prolifération, de différentiation et d'apoptose finement régulées par les hormones et les facteurs de croissance. Les interactions cellulaires sont également nécessaires à ce processus et leur régulation est liée aux taux hormonaux dans plusieurs tissus. Ainsi, toute perturbation dans ce système peut entrainer des anomalies développementales, un dysfonctionnement de la glande et même être associée au cancer du sein.

Les retardateurs de flammes bromés : des perturbateurs endocriniens omniprésents

Les perturbateurs endocriniens (EDs) sont les substances exogènes qui perturbent la fonction naturelle des hormones par diffèrents mécanismes (Mallozzi, Bordi et al. 2016). Il a été démontré qu'une exposition à certains EDs à des périodes sensibles du développement des glandes mammaires, telles que la période périnatale, la puberté ou la grossesse, peut engendrer des anomalies développementales et favoriser la formation de tumeurs (Paulose, Speroni et al. 2015) (Soto, Vandenberg et al. 2008).

Les retardateurs de flammes bromés (BFRs) sont un groupe de molécules qui sont ajoutées dans divers matériaux afin de diminuer leur inflammabilité (G. Camino 1991, Watanabe and Sakai 2003). Cependant, puisqu'ils ne forment pas de liens covalents avec les matrices auxquelles ils sont ajoutés, les BFRs sont facilement relâchés dans l'environnement, générant ainsi une exposition humaine continue (Besis and Samara 2012). Bien que l'utilisation, la production et l'importation de certains congénères de BFRs soient maintenant interdites au Canada, l'exposition humaine continuera vraisemblablement pour plusieurs années puisque les BFRs sont bioaccumulables et persistants, et qu'ils sont toujours relâchés d'items existants (Environment 2008) (Environment 2010) (Dodson, Perovich et al. 2012). Il a été démontré que certains BFRs agissent comme des perturbateurs endocriniens (Paulose, Speroni et al. 2015). Étant donné que des niveaux significatifs ont été quantifiés dans le lait maternel, les tissues adipeux et les glandes mammaires (Toms, Hearn et al. 2009) et que le développement et la fonction des glandes mammaires sont finement orchestrés par les hormones, une exposition à des BFRs pourrait avoir des effets importants pour sur ces organes.

Hypothèses et objectives de recherche

Plusieurs points importants peuvent être tirés de cette revue de la littérature :

1. La glande mammaire est un organe dynamique qui subit plusieurs changements substantiels au cours de la vie.
2. Les jonctions intercellulaires sont nécessaires aux interactions entre les cellules épithéliales de la glande mammaire.
3. Les protéines des jonctions gap, adhérentes et serrées interagissent et forment un nexus entre les cellules adjacentes.
4. Une inhibition, une dérégulation ou une délocalisation des protéines jonctionnelles peuvent engendrer des problèmes développementaux et sont impliquées dans la cancérogenèse.
5. Le développement des glandes mammaires est finement orchestré par les hormones et les facteurs de croissance.
6. L'expression des protéines jonctionnelles est également régulée par les hormones.
7. Les perturbateurs endocriniens sont définis comme des molécules perturbant l'action normale des hormones.
8. Les retardateurs de flammes bromés sont des polluants hautement répandus dans l'environnement, et certains sont considérés comme des perturbateurs endocriniens.

En se basant sur ces faits, nous avons émis l'hypothèse que la composition du nexus jonctionnel varie selon les différents stades de développement des glandes mammaires, et qu'une exposition à des perturbateurs endocriniens pourrait déréguler l'expression des protéines jonctionnelles, engendrant ainsi des anomalies développementales ou fonctionnelles.

Les deux principaux objectifs, et les sous-objectifs spécifiques, de ce projet de recherche étaient donc de :

A. Caractériser la dynamique du nexus jonctionnel durant les différentes phases de développement des glandes mammaires en :
    a. Déterminant l'expression des protéines des jonctions gap, adhérentes et serrées aux différents stades de développement;
    b. Identifiant les protéines présentes dans le nexus jonctionnel selon les stades de développement;

c. Confirmant les interactions physiques entre les différentes protéines du nexus.

Les résultats des expériences liées à cet objectif ont été publiés dans deux articles (voir chapitre 2) :

i. 1ière publication; Dianati E, Poiraud J, Weber-Ouellette A and Plante I, *Connexins, E-cadherin, Claudin-7 and β-catenin transiently form junctional nexuses during the post-natal mammary gland development*, Developmental Biology, 2016 Aug 1;416(1):52-68. doi: 10.1016/j.ydbio.2016.06.011. Epub 2016 Jun 9.

ii. 2ième publication; Dianati E and Plante I, *Analysis of protein-protein interactions and co-localisation between components of gap, tight and adherens junctions in murine mammary glands*, Journal of Visualized Experiments (sous presse).

B) Déterminer les effets d'une exposition à un mélange de retardateurs bromés sur le développement des glandes mammaires et sur leur fonction, en :

a. Évaluant la fonctionnalité de la glande mammaire, telle que définie par la synthèse du lait et les voies de signalisation qui y sont associées.
b. Déterminant l'expression et la localisation de marqueurs de la transition épithélio-mésenchymale et des protéines jonctionnelles.
c. Caractérisant les rôles de β-caténine en tant que membre des jonctions adhérentes et en tant que facteur de transcription.

Les résultats liés à cet objectif sont regroupés dans une publication (voir chapitre 2), et une seconde, effectuée en collaboration, est en préparation pour soumission (voir annexe):

i. 1ière publication; Dianati E, Wade M, Hales B, Robaire B and Plante I, Exposure to an Environmentally Relevant Mixture of Brominated Flame Retardants Decreased p-β-catenin^{ser675} Expression and its Interaction with E-cadherin in the Mammary Glands of Lactating Rats (re-soumis après correction à Toxicological Sciences)

ii. 2ième publication; Lavoie M, Dianati E, Wade M, Hales B, Robaire B and Plante I, *A Perinatal Exposure to Brominated Flame Retardants Suppresses E-cadherin and Hormone Receptor α Expression in Mammary Glands at Puberty* (titre non-officiel, manuscrit en préparation)

Méthodologies

Les modèles animaux

Objectif 1

Afin de caractériser la dynamique du nexus jonctionnel durant les différentes phases de développement des glandes mammaires, des souris C57BL6 femelles ont été sacrifiées à des stades clés du développement. Les stades suivants ont été étudiés :

- Les souris vierges (avant puberté (W4), au moment de la puberté (W6) et adultes (W10))
- Les souris gestantes (au milieu (P8) et à la fin de la gestation (P18))
- Les souris en lactation (première (L7) et deuxième semaine de la lactation (L14))
- Les souris en sevrage (phase réversible (In1) et irréversible (In8))

Objectif 2

Afin de déterminer les effets d'une exposition à un mélange de retardateurs bromés (BFRs) sur le développement des glandes mammaires et sur leur fonction, des rats femelles (Sprague-Dawley) ont été exposés par la diète à un mélange de BFRs avant l'accouplement, pendant la grossesse et pendant l'allaitement, et sacrifiées au jour du sevrage (jours 21). Les bébés ont aussi été sacrifiés aux jours 21, 46 et 100 post-natal afin d'étudier les effets d'une exposition périnatale à des BFRs sur le glande mammaire (voir annexe et les autres articles publiés par nos collaborateurs (Berger, Lefevre et al. 2014, Lefevre, Berger et al. 2016, Lefevre, Wade et al. 2016). La formulation du mélange de BFRs est basée sur les proportions des différents congénères (trois PBDE : DE-71, DE-79 et BDE-209, et HBCD) qui ont été mesurées dans la poussière de maison (Allen, McClean et al. 2008). Les diètes ont été préparées afin de délivrer les doses nominales de 0, 0.06, 20 and 60 mg/kg poids de corps/jour. La dose faible reflète l'exposition humaine en Amérique Nord (Allen, McClean et al. 2008).

Pour chacune des souris ou chacun des rats, les glandes inguinales et abdominales (#4-5) ont été coupées en morceaux, congelées directement dans l'azote liquide et conservées à -80, tandis que les glandes thoraciques (#2-3) ont été enrobées dans la paraffine (côté gauche) ou dans la cryomatrice (côté droit).

Analyses effectuées

Pour chaque souris ou rat, un morceau provenant des glandes inguinales et abdominales a été homogénéisé afin de quantifier les niveaux protéiques des protéines d'intérêts par analyses d'immunobuvardage de type western. Ces homogénats ont également servit à analyser les interactions cellulaires par co-immunoprécipitation. La méthode d'immunoprécipitation est décrite en détails dans le deuxième article. Un autre morceau des glandes a servi à isoler l'ARN afin de quantifier les transcrits par RT-qPCR.

Pour chaque souris ou rat, les glandes thoraciques droites ont été enrobées dans la paraffine et des coupes histologiques ont été réalisées. Ces coupes ont été colorées à l'aide de la méthode Trichrome Masson et examinées en lumière blanche. Les glandes thoraciques gauches ont été enrobées dans la cryomatrice et les cryosections ont été utilisées pour déterminer la localisation des protéines d'intérêts, tel que décrit dans la deuxième publication.

Les principaux résultats

Note : pour cette section, la numérotation des figures réfère à leur numéro dans chacun des articles; un code de couleur a été adopté (rouge pour les figures du premier article, vert pour le deuxième article et mauve pour le troisième article).

La publication 1) Les connexines, E-cadhérine, Claudine-7 et β-caténine forment un nexus jonctionnel transitoire au cours du développement des glandes mammaires

Les expériences présentées dans cet article avaient pour but de déterminer si les Cxs interagissent avec les protéines des TJs et des AJs dans les glandes mammaires, et de caractériser l'expression et la localisation des différentes protéines, ainsi que la composition des complexes jonctionnels.

En utilisant de la coloration Masson's trichrome, nous avons d'abord montré que la structure des glandes est effectivement représentative des différents stades de développement des glandes mammaires (Figure 1). Afin d'évaluer l'expression des jonctions intercellulaire, les glandes mammaires ont été homogénéisées, et les protéines et transcrits de différentes protéines jonctionnelles ont été mesurés par les techniques de RT-qPCR et de western blot. Nos résultats ont démontré que les niveaux d'ARNm et de protéines des Cx26, Cx30 et Cx32, varient au cours du développement des glandes mammaires (Figures 2B-D et 3B-D). D'un autre côté, les niveaux d'ARNm et de protéines de la Cx43 sont

détectables à tous les stades de développement, et démontrent les plus faibles niveaux d'expression pendant la lactation (Figure 2A-B). Ces résultats suggèrent que l'expression de chaque Cx varie selon les besoins fonctionnels du tissu et est contrôlée par différents mécanismes, probablement liés aux niveaux hormonaux.

Afin de vérifier si des composantes des AJs et TJs sont exprimées de façon concomitante au Cxs, nous avons ensuite vérifié leurs niveaux d'expression protéiques et en ARNm. Nos résultats ont démontré que l'expression des claudine-1, -3, -4 et -7, ainsi que de E-cadhérine, β-caténine et P-cadherine suivent une même tendance : elles augmentent graduellement entre W4 et P8, et de façon plus importante à la fin de la gestation, diminuent pendant la lactation et s'élève à nouveau lors de l'involution (Figures 4 et 6). Cependant, les niveaux protéiques différaient quelques peu. Bien que les transcrits de claudine-3 et -7 diminuent pendant la lactation, les protéines sont toujours exprimées; de même, les niveaux protéiques de claudine-1 demeurent relativement stables, suggérant l'existence d'un mécanisme de stabilisation pour ces protéines. Ces résultats suggèrent un rôle important joué par les claudines à ces stades du développement (Figures 4 et 5).

Nos résultats ont démontré que l'expression protéique de E-cadhérine et β-caténine est à son plus haut à la fin de la gestation, suivit d'une diminution graduelle. Par contre, les niveaux protéiques de P-cadhérine augmentent entre W10 et P8, redescendent en fin de gestation, puis remontent graduellement à partir de L14. Ces résultats suggèrent que la régulation des protéines des AJs est différente entre les cellules myoépithéliales et luminales (Figures 6 et 7).

Puisque nos résultats démontraient que plusieurs protéines des GJs, AJs et TJs sont exprimées aux mêmes stades de développement, nous avons ensuite voulu vérifier leur localisation par immunofluorescence. Nos analyses ont démontré que Cx43, Cx26, Cx30 et Cx32 sont majoritairement localisées en plaques à la membrane plasmique des cellules, un pattern typique pour les GJs (Figure 8). Tel que prévu, Cx43 est exprimée dans les cellules myoépithéliales, tandis que Cx26, Cx30 et Cx32 sont exprimées dans les cellules luminales, mais seulement à certains stades (Figure 8). Une faible expression de Cx43 a également été observée entre les cellules luminales ou à l'interface entre les cellules luminales et myoépithéliales (Figure 8E). Claudine-3, -4 et -7 sont localisées à la membrane au niveau basolatéral au cours du développement mammaires, mais de façon plus apicale pendant la gestation et la lactation (Figure 9). E-cadhérine est majoritairement localisée à la membrane jusqu'à P18, bien qu'une localisation cytoplasmique soit observée pendant la lactation (Figure 9 et sup Fig 2). β-caténine est

toujours présente à la membrane de façon basolatérale et apicalee au cours du développement mammaire (Figure 9). P-cadhérine est localisée autour des cellules myoépithéliales ou dans le stroma (Figure 9).

L'ensemble de ces résultats démontrent que certaines composantes des AJs et TJs ont des patrons d'expression et de localisation similaires aux Cxs, et pourraient donc interagir ensemble. Afin de vérifier cette hypothèse, la technique de l'immunoprécipitation a été utilisée. Nos résultats démontrent que Cx43 et β-caténine interagissent à tous les stades de développement. De même, Cx43 et claudine-7 interagissent à tous les stades, mais cette interaction est plus forte à la fin de la gestation. Cx43 et E-cadhérine interagissent surtout à la fin de la gestation, pendant la lactation et au début de l'involution (Figure 10A).

Aucune interaction n'a été démontrée entre Cx30 et les composantes des AJs et TJs. Par contre, Cx30 interagit avec Cx26 à la fin de la gestation et au début de la lactation (Figure 10B). Cx32 interagit également avec Cx26, mais aussi avec E-cadhérine et β-caténin pendant la lactation (Figure 10C).

L'ensemble de ces résultats a permis de confirmer la présence des nexus jonctionnels dynamiques dont les composantes provenant des GJs, AJs et TJs varient pendant le développement des glandes mammaires. L'ensemble de nos résultats suggèrent que l'expression des Cxs peut être régulée, du moins en partie, par des interactions protéiques avec les protéines des TJs et AJs.

La publication 2) Analyse des interactions protéine-protéine et de co-localisation entre les composants des jonctions gap, serrées et adhérents dans les glandes mammaires murines

Cet article présente une combinaison de méthodes permettant de démontrer des interactions entre les protéines jonctionnelles dans la glande mammaire. La première partie de cet article explique comment vérifier la co-localisation de potentiels partenaires d'interaction à l'aide de l'immunofluorescence. La deuxième partie consiste en un protocole détaillé de la méthode de co-immunoprécipitation afin de démontrer des liens physiques entre les protéines.

La partie « résultats » présente des exemples d'analyses possibles en utilisant ces techniques. Tout d'abord l'immunofluorescence a permis de démontrer que la β-caténine et Cx26 co-localisent à la membrane plasmique des cellules luminales dans la glande mammaire en lactation (Figure 1). Par la suite, nous avons démontré qu'il est possible d'observer la co-localisation de trois protéines en utilisant de l'immunofluorescence. Nos résultats montrent que Cx26, claudine-7 et E-cadhérine co-localisent à la

membrane plasmique des cellules luminales dans les glandes mammaires en fin de gestation (Figure 2). Finalement, des analyses d'immunoprécipitation ont permis de déterminé que Cx43 interagit avec E-cadhérine et claudine-7, mais pas avec claudine-3 (Figure 3).

Les résultats présentés illustrent qu'il est possible de démontrer des interactions entre des protéines jonctionnelles dans les glandes mammaires en utilisant les protocoles décrits. Bien qu'il existe d'autres méthodes permettant de vérifier les interactions protéines-protéines, l'immunofluorescence et la co-immunoprécipitation demeurent des méthodes fiables et relativement peu couteuses.

La publication 3) Une exposition à un mélange de Retardateurs de Flammes Bromés diminue l'expression de p-β-Caténine^{Ser675} et inhibe l'interaction entre p-β-Caténine^{Ser675} et E-cadhérine dans les glandes mammaires de rats

Les expériences présentées dans cet article avaient pour but de déterminer les effets d'une exposition à un mélange de BFRs pendant la grossesse et la lactation sur la fonction de la glande mammaire et sur les protéines jonctionnelles.

L'exposition aux BFRs n'a pas affecté le poids des glandes mammaires (Figure supplémentaire 1A), l'expression de protéines liées à la synthèse du lait (Stat5, p-Stat5^{tyr694}, β-caséine; Figure supplémentaire 1B-C) ou l'expression des récepteurs hormonaux étudiés (EGFR/ErbB-1, pEGFR (Tyr1068), ErbB2, ERα et récepteurs des hormones thyroïdiennes α et β; Figure supplémentaire 2A-F). Ces données suggèrent que le traitement n'affecte pas la lactation.

Par la suite, nous avons étudié la double fonction de β-caténine, soit son rôle dans les AJs et son rôle en tant que facteur de transcription. Bien que les niveaux totaux de β-caténine, les niveaux de non-p-β-cat^{Ser45} et de p-β-cat^{Tyr654} (Figure 1A,B,D) fussent inchangés, une baisse significative d'environ 50% de la forme phosphorylée sur la sérine 675 de la β-caténine (p-β-cat^{Ser675}) a été observée dans le groupe exposé à la dose de 0.06 mg/kg (Figure 1C). Nos résultats ont démontré que cette baisse n'était vraisemblablement pas due à une baisse d'activité d'Akt et de GSK-3β puisqu'il n'y avait aucun changement dans leur expression ou celle de leurs formes phosphorylées (p-Akt^{Ser473} et p-GSK-3β^{Ser9} (Figure 2A-D). Par contre, nos résultats suggèrent que la baisse p-β-cat^{Ser675} pourrait être liée à une baisse d'activité de la protéine kinase A (PKA), et non à une baisse de ces niveaux (Figure 2E), puisque l'expression de la forme phosphorylée de CREB1, une cible directe de PKA, étaient également diminuée

chez les animaux traités avec les doses de 0.06 et 30 mg/kg, sans que les niveaux de CREB soient affectés (Figures 3F, G).

Nous avons par la suite voulu déterminer si la baisse de p-β-cat^{Ser675} affectait les fonctions de β-caténine en tant que facteur de transcription. Aucune interaction n'a été démontré entre p-β-cat^{Ser675} et LEF1, le co-facteur de transcription de β-caténine (Figure 3A), et aucun changement n'a été observé dans les niveaux d'expression des gènes cibles de β-caténine/LEF1 TCF1, c-Jun, c-Myc et Met (Figures 3B-E).

Nous avons ensuite évalué si la baisse de p-β-Cat^{Ser675} affectait les fonctions de β-caténine en tant que membre des AJs. Nos résultats en immunofluorescence ont démontré que p-β-cat^{Ser675}, tout comme β-caténine, est localisée à la membrane cytoplasmique des cellules luminales, et que son expression est diminuée chez les animaux traités avec les doses faibles et moyennes de BFRs (Figure 4A-D). Par contre, aucun différence n'a été observée dans les niveaux protéiques ou la localisation des autres protéines des GJs, TJs et AJs étudiées, ni dans les niveaux de marqueurs de la transition épithélio-mésenchymateuse (Figure 5A-H et Figure supplémentaire 3). Finalement, nos résultats ont démontré une diminution significative de l'interaction entre les p-β-cat^{Ser675} et E-cadhérine chez les animaux traités avec la faible dose de BFRs (Figure 6A-C).

L'ensemble de ces résultats démontrent qu'une exposition à un mélange de BFRs à une dose représentative de l'exposition humaine a peu d'effets sur la fonction de la glande mammaire, mais affecte le niveau protéique de la p-β-cat^{ser675} et son interaction avec E-cadhérine, vraisemblablement par un mécanisme lié à l'inactivation de PKA. Ces résultats suggèrent qu'une exposition à un mélange de BFRs modifie la signalisation dans la glande mammaire. Les conséquences à plus long terme de ces changements restent à être déterminées.

Discussion et conclusion

Les glandes mammaires sont en constant remodelage au cours de la vie des mammifères. Bien qu'une dérégulation des niveaux d'expression ou de la localisation des composantes des GJs ait été associée à des anomalies développementales et au cancer du sein, les mécanismes impliqués dans leur régulation sont peu connus. Dans plusieurs tissus, les Cxs interagissent et forment un complexe jonctionnel avec les protéines des AJs et TJs, ce qui stabiliserait leur activité, et qui suggère l'existence de mécanismes de régulation communs. Ainsi, déterminer l'expression, la localisation et les interactions des protéines jonctionnelles au cours du développement est crucial afin de bien comprendre le rôle joué par les Cxs

dans le développement normal des glandes mammaires, et dans les pathologies. Notre étude est un premier pas dans cette direction. Elle comportait deux objectifs : caractériser le complexe jonctionnel dans les différents stades de développement des glandes mammaires, et déterminer si une exposition à un mélange de BFRs pouvait perturber le développement de la glande mammaire en dérégulant les interactions cellulaires.

Les protéines des jonctions gap, serrées et adhérentes forment un complexe jonctionnel dynamique durant le développement de la glande mammaire

Notre étude a permis de démontrer que les Cxs forment des complexes jonctionnels qui varient de façon stade-dépendante. Les interactions entre Cx43 et β-caténine et entre Cx43 et E-cadhérine ont été déjà démontrées dans différente tissus (Jongen et al., 1991; Hernandez-Blazquez et al., 2001) (Ai et al., 2000; Shaw et al., 2007) (Ale-Agha et al., 2009; Fujimoto et al., 1997). Il avait alors été suggéré que ces interactions étaient impliquées dans la stabilisation, la formation et le positionnement des jonctions à la membrane plasmique. Nos études ont également démontré, et ce pour la première fois, une interaction entre Cx43 et claudine-7, et que cette interaction était plus forte à la fin de la gestation. Cx43 est majoritairement exprimée dans les cellules myoépithéliales, et faiblement dans les cellules luminales, tandis que claudine-7 est exprimée dans les luminales. Au moins deux explications, qui ne sont pas exclusives, peuvent supporter ces résultats : l'interaction entre Cx43 et claudine-7 se produit 1) à la jonction entre les cellules myoépithéliales et les cellules luminales, ou 2) dans les cellules luminales. Des études supplémentaires sont nécessaires afin de vérifier ces deux hypothèses. Nos résultats ont également démontré une interaction entre Cx32 et E-cadhérine, ainsi qu'entre Cx32 et β-caténine. La co-localisation de Cx32 et β-caténine a déjà été rapportée (Schwarz et al., 2003; Kanczuga-Koda et al., 2014; Ionta et al., 2012; Plante et al., 2005, 2006). Ces interactions entre les composantes de différents types de jonctions intercellulaires pourraient jouer un rôle dans la régulation de leur fonction mutuelle, mais aussi permettre des échanges différents en fonction du stade de développement de la glande mammaire. De plus, ces résultats suggèrent l'existence de mécanismes régulateurs communs aux différentes composantes des jonctions.

Nous avons également démontré des interactions entre Cx32 et Cx26, et entre Cx30 et Cx26 pendant la lactation et à la fin de la gestation, respectivement. Ces résultats confirment la présence de canaux de GJs hétérotypiques ou de connexons hétéromériques à ces stades du développement. Puisqu'il a été démontré que la composition des canaux influence les molécules qui peuvent passer

d'une cellule à l'autre, la présence de ces canaux suggère des besoins particuliers à ces stades. D'un autre côté, il a été démontré qu'une inhibition de Cx26 pendant la grossesse et la lactation n'affecte pas la formation des alvéoles ni les capacités de lactation des souris (Stewart et al. 2015). Il est donc possible que la présence de Cx32 et Cx30 compense pour cette perte. Des recherches supplémentaires sont nécessaires pour déterminer le rôle et les mécanismes régulant ces interactions dans le développement mammaire normal, ainsi que leurs contributions aux pathologies et le cancer du sein.

L'étude des interactions protéines-protéines dans les glandes mammaires

Plusieurs mises au point ont été nécessaires pour réaliser les expériences permettant de déterminer les interactions protéines-protéines dans les glandes mammaires. Nous avons donc publié un article décrivant ces méthodes. Bien que les méthodes aient été optimisées pour étudier les jonctions cellulaires dans les glandes mammaires des rongeurs *in vivo*, elles peuvent être utilisées dans n'importe quel tissu et pour d'autres types de protéines suite à de légères optimisations. De plus, bien que de nouvelles techniques aient été développées pour évaluer les interactions protéines-protéines, les méthodes que nous avons présentées dans cet article sont généralement moins chères et les plus facilement réalisable, et peuvent être utilisées *in vivo.*

Une exposition à un mélange de BFRs reflétant l'exposition humaine affecte la phosphorylation de β-caténine et son interaction avec E-cadhérine

Puisque le développement et la fonction de la glande mammaire sont dépendants des hormones, un dérèglement de l'équilibre hormonal peut influencer la structure et la physiologie de la glande mammaire et perturber la lactation. Il a été démontré que certains BFRs agissent comme les perturbateurs endocriniens. Par contre, aucune étude n'avait, jusqu'à maintenant, évalué leurs effets sur la glande mammaire chez la femelle gestante et en lactation.

Notre étude a démontré que les niveaux de p-β-cat^{ser675} étaient diminués de façon significative et concomitante à une baisse de p-CREB. Ces résultats suggèrent une baisse de l'activité PKA dans les animaux traités à la plus faible dose. Le lien entre PKA et p-β-cat^{ser675} avait déjà été établi dans d'autres tissus, mais jamais dans la glande mammaire (Taurin, Sandbo et al. 2006, Spirli, Locatelli et al. 2013) (Law, Weck et al. 2013) (Cai, Sun et al. 2016) (Cai et al., 2016) (Estus, Choudhary et al. 2016). Cependant, la phosphorylation de β-caténine ne semble pas modifier sa localisation, ni activer l'expression de gènes cibles du complexe β-caténine/LEF1, tel qu'il avait été démontré auparavant (Taurin, Sandbo et al. 2006,

Spirli, Locatelli et al. 2013). Jusqu'à maintenant, le lien entre p-β-cat^{ser675} et une augmentation de l'activité transcriptionnelle n'avait été démontré que dans des études *in vitro*, et toujours avec des cellules préalablement stimulées (Taurin, Sandbo et al. 2006, Spirli, Locatelli et al. 2013). Nos résultats démontrent qu'*in vivo*, dans la glande mammaire en lactation, p-β-cat^{ser675} est localisée à la membrane plasmique, où elle interagit avec E-cadhérine. Une baisse de l'association entre E-cadhérine et β-caténine a été associée à un mauvais pronostic pour le cancer du sein (Dolled-Filhart et al., 2006). Il est donc crucial de comprendre les conséquences à long-terme de la baisse de p-β-cat^{ser675} dans la glande mammaire.

Les résultats de ce deuxième objectif démontrent qu'une exposition à un mélange de BFRs n'a pas d'effets visibles sur la structure et fonction de la glande mammaire pendant la lactation. Cette exposition inhibe la liaison de β-caténine et E-cadhérine à la membrane plasmique, vraisemblablement par un mécanisme lié à l'inactivation de PKA. Cette baisse est significative à la plus faible dose, soit celle qui est la plus représentative de l'exposition humaine, puisqu'elle est basée sur les proportions et les niveaux des différents congénères retrouvés dans la poussière de maison. Des recherches supplémentaires sont nécessaires afin de mieux comprendre les conséquences de ces changements à plus long terme.

Conclusion

L'ensemble de nos résultats démontrent que les connexines sont associées à différents membres des AJs et des TJs dans un complexe jonctionnel dynamique durant le développement de la glande mammaire. L'expression des différentes composantes des GJs, AJs et TJs varient également selon les stades de développement, suggérant l'existence de mécanismes de régulation communs, vraisemblablement liés aux changements hormonaux. Cette hypothèse est également appuyée par le fait qu'une exposition à des produits démontrant un potentiel de perturbateurs endocriniens inhibe la liaison entre deux membres importants du complexe jonctionnel. Il sera donc important de mieux caractériser la régulation des protéines jonctionnelles lors du développement de la glande mammaire afin de comprendre comment leur dérégulation peut être liée à des anomalies développementales et au cancer du sein.

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1. Introduction

1.1 Mammary gland organogenesis and development;

The mammary gland (breast) differentiates the mammals from other animals. It is the organ which is responsible for nourishing the mammals' newborns by secreting the milk during the nursing period. Organogenesis is the formation and development of the organs of living things. Generally organs development is the last step of embryonic development from three partitions of germ layers. Although, mammary gland development starts before birth but mostly occurs during adolescence (Macias and Hinck 2012) (Richert, Schwertfeger et al. 2000).

1.1.1 Pre-natal mammary gland development

In mice, at embryonic day 10.5 (E10.5), mammary gland development begins with generation of two bilateral epidermal ridges also called milk lines (Figure 1). Two ridges on each side of the embryo cover the ventral surface from forelimb to hindlimb. At E11.5, five pairs of disk shaped placodes will be segregated from ridges at the sites of future nipples (Hens and Wysolmerski 2005) (Sternlicht 2006). These placodes will be formed in an orderly process beginning from thoracic pairs 3, followed by abdominal pairs 4 and inguinal pairs 5 and finally with cervical pairs 1 and upper thoracic pairs 2 (Veltmaat, Mailleux et al. 2003). Then, at E12.5, ridges grow into the underlying mesenchyme and form the bulb shaped buds. The mesenchymal cells will arrange in layers around the epithelial ridges and this structure represents the first mammary analge. At E14.5, the gender difference will occur. While in females mesenchymal cells will be surrounding mammary buds, in males they will condense around the bud and constrict mesenchyme connection with ridge and gradually lead to major apoptosis in both compartments. In females at E16, intercellular space between the ridges and lumen will start to form; at E18 a distinct lumen will be apparent. At E18.5, mammary buds will enter the lower dermis and grow between dermis and fat pad. During this process, ductal branching will occur 1-20 times (Hens and Wysolmerski 2005) (Sternlicht 2006) (Richert, Schwertfeger et al. 2000) (Robinson 2007). Elongation will resume and this rudimentary ductal tree will be present at the time of birth.

In humans, several sprouts will be extended from the mammary buds, creating multiple mammary trees that will join together at the nipple. Nipple will be generated by the thickening of epidermis overlying the primary mesenchyme. Consequently nipple sheet will be generated from keratinocytes (Sternlicht 2006) (Macias and Hinck 2012) (Richert, Schwertfeger et al. 2000) (Robinson 2007).

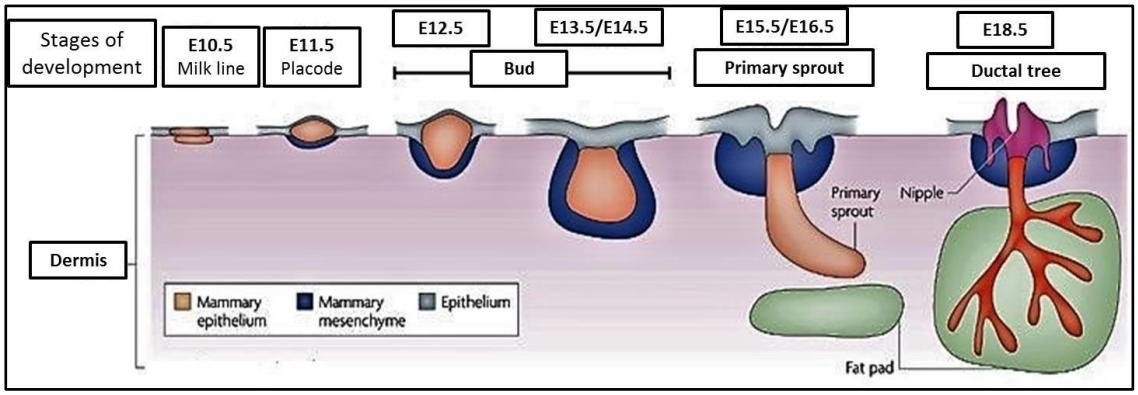

Figure 1) Embryonic mammary gland development. Schematic view of pre-natal mammary gland development (Robinson 2007).

1.1.2 Post-natal mammary gland development

From the birth time until puberty, mammary gland is at dormant phase. At the time of puberty, around 6 weeks old in mice, under the surge of hormones released by ovaries, cells from the rudimentary ductal tree undergo proliferation and form multicellular club shaped terminal end buds (TEBs) at the end of ducts (Figure 3) (Sternlicht, Kouros-Mehr et al. 2006) (Sternlicht 2006). TEBs consist of an outer layer of cap cells, which become myoepithelial cells, and an inner layer of body cells which will form the luminal cell compartment (Sternlicht 2006). As proliferation continues, ducts will elongate and further penetrate into the fat pad (Sternlicht 2006).

At adult stage, around 10 weeks old in mice, TEBs are no longer observable and most of the fat pad is invaded by the epithelium (Figure 3). The rest of the space will be taken by adipose tissue, blood vessels, immune cells, fibroblasts and stem cells (Daniel CW 1987) (Atwood, Hovey et al. 2000) (Neville, McFadden et al. 2002).

Across the mammals life span, at each estrous cycle tertiary branches will develop (Figure 3), mainly following the rise of progesterone levels, and prepare the organ for the potential gestation (Sternlicht, Kouros-Mehr et al. 2006) (Sternlicht 2006).

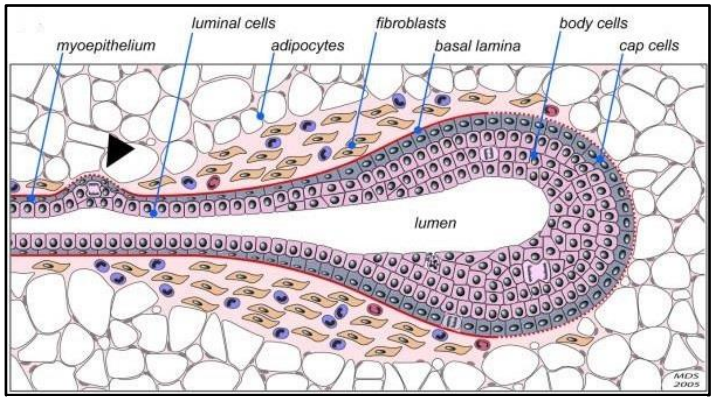

Figure 2) Terminal End Buds (TEBs) structure. (Sternlicht 2006).

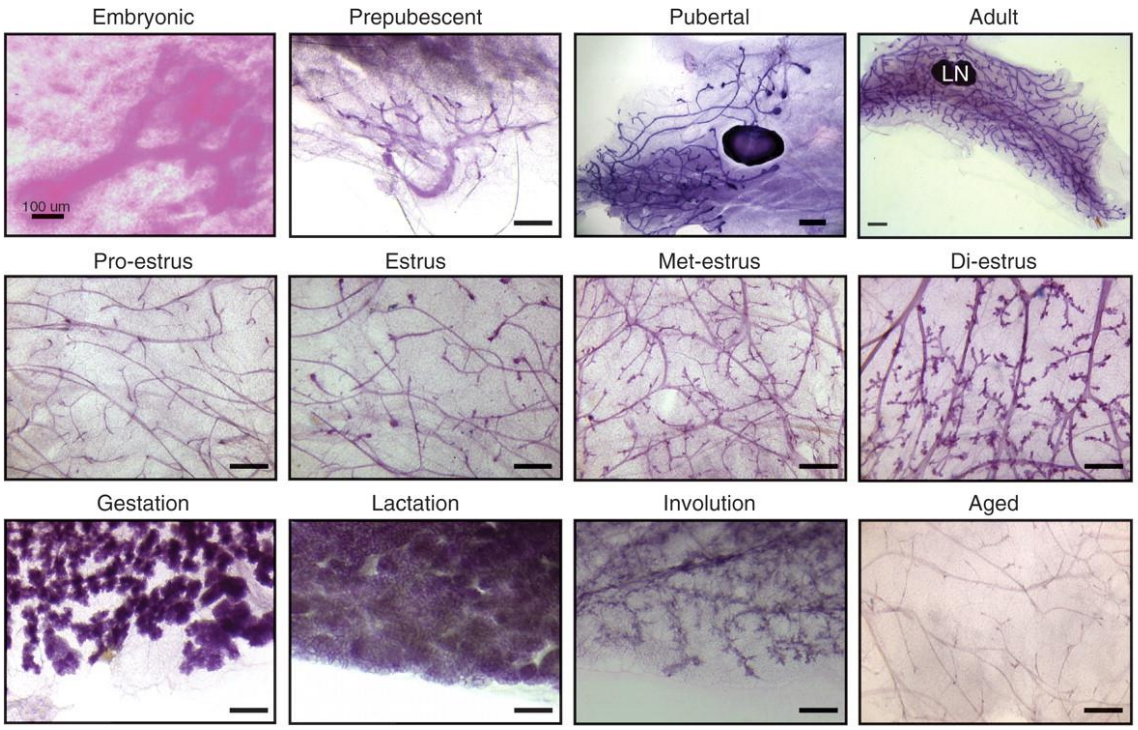

Figure 3) Murine post-natal gland development. Whole mount of mammary glands (CSH).

Mammary glands only acquire their final development stage during gestation and lactation (Figure 3). At pregnancy, mainly under the influence of prolactin and placental lactogen, the elongated

ducts from lateral sides start branching in a dichotomous manner filling the fat pad with alveolar buds. Epithelial cells proliferate and generate the alveolar buds which differentiate into alveoli, the milk secreting units (Oakes, Hilton et al. 2006) (Daniel CW 1987) (Atwood, Hovey et al. 2000) (Neville, McFadden et al. 2002) (Richert, Schwertfeger et al. 2000) (Sternlicht 2006).

At parturition, alveolar epithelial cells will be responsible for milk productions and secretion to the lumen. During the lactation, while the luminal alveolar cells are responsible for milk synthesis and secretion to the lumen, milk expulsion toward the ducts occurs mainly by contractility of myoepithelial cells surrounding the alveoli. The duration of gestation and lactation is about 3 weeks in mice (Oakes, Hilton et al. 2006) (Daniel CW 1987) (Atwood, Hovey et al. 2000) (Neville, McFadden et al. 2002) (Richert, Schwertfeger et al. 2000) (Sternlicht 2006).

After weaning, the gland will undergo a stage of regression named involution (Figure 3). (Macias and Hinck 2012). Involution is comprised of a reversible and an irreversible step (Sternlicht 2006) (Li, Liu et al. 1997) (Marti, Feng et al. 1997, Macias and Hinck 2012). The lack of demand or suckling at involution day 1 leads to lysosomal proteases activation, causing lysosomal membrane permeabilization, and removal of milk producing parenchyma. When separation of pups is prolonged for more than 12 hours, apoptosis will lead to alveolar cell detachment and shedding in to the lumen. These processes can be reversed upon resumption of suckling. However, after 48 hours without milk demand, the irreversible phase of involution starts with alveoli collapsing, loss of milk supply and a second phase of apoptosis. Finally, at involution day 8, the structure of the glands totally regresses back to the simple ductal structure similar to adult phase (Li, Liu et al. 1997) (Marti, Feng et al. 1997, Macias and Hinck 2012).

Two main regulators of involution are the serine proteases, which convert plasminogen to plasmin, and matrix metalloproteinases (MMPs), which initiate extracellular matrix (ECM) disruption. During the first phase of involution, plasmin causes the loss of interactions between epithelial cells and ECM (Li, Liu et al. 1997) (Marti, Feng et al. 1997, Macias and Hinck 2012). In the second phase, combinatory effect of plasmin and MMPs disrupt the basal lamina. Moreover, these enzymes are crucial for releasing growth and differentiation factors which are required for replacement of adipocytes when epithelial tissue loss occurs (Li, Liu et al. 1997) (Daniel CW 1987, Marti, Feng et al. 1997, Macias and Hinck 2012) (Atwood, Hovey et al. 2000) (Neville, McFadden et al. 2002) (Richert, Schwertfeger et al. 2000) (Sternlicht 2006).

There is only one pair of mammary glands in human, 5 in mice and 6 in rats (Macias and Hinck 2012). The epithelium (or parenchyma) of mice and rat glands are similar to human as the gland architecture and remodeling is highly conserved. However, there are some differences in terms of structural units: the rodent parenchyma consists of one elongated ductal tree for each nipple while multiple ductal networks are present in human. Moreover, the relative abundance of connective tissue in the stroma around the parenchyma is considerably different in mouse and human (Maller, Martinson et al. 2010, Inman, Robertson et al. 2015). In mice, white adipose tissue is abundant while the stroma surrounding the lobules and ducts is sparse and there is little fibrous tissue between ducts. In human, little white adipose tissue is present, but there is an abundant stroma around the ducts and alveoli as well as a greater number of fibrous connective tissue between ducts. Another important difference between mice and human is that in mice, the male rudiment will be destroyed during embryonic development, while in humans male and female breast undergo the same changes until puberty, and can even be prolonged in male breast malignancy (Maller, Martinson et al. 2010, Inman, Robertson et al. 2015) (Tiede and Kang 2011). Moreover, in human breast the main lobular units are terminal ductal lobular unit (TDLUs) which are termed Lob1-type is equivalent to TEBs in rodents. Lob-1-type TDLUs begins to differentiate to Lob-2 generating the ductal structure and then Lob-3 during pregnancy forming ductules and eventually Lob-4 forming secretory acinar structures. After menopause, human breast is again comprised of Lob-1 structures (Tiede and Kang 2011) (Sternlicht, Kouros-Mehr et al. 2006) (Maller, Martinson et al. 2010).

1.2 Mammary gland structure;

The adult mammary gland is comprised of multiple cell types. In adult virgin mice, the epithelium is composed of luminal epithelial cells around the lumen which are surrounded by a basal layer, mainly composed of myoepithelial cells (Figure 4) (Maller, Martinson et al. 2010, Inman, Robertson et al. 2015) (Tiede and Kang 2011). During puberty, cap cells of the TEBs contact the stroma and generate the thick basal lamina, and the body cells fill the interior of the buds. The central body cells go through apoptosis and generate the lumen while the surrounding body cells differentiate into luminal epithelial cells (Figure 4). During pregnancy, luminal epithelial cells proliferate, expand and generate the alveolar cells responsible of milk secretion (Figure 4) (Inman, Robertson et al. 2015) (Visvader 2009). The mammary glands epitheliums also contain stem cells which provide the dramatic regenerative potential at each cycle of growth and involution. Mammary stem cells (MaSCs) can be

bipotent rising both luminal and myoepithelial cells or be unipotent which just give rise to a single lineage (Tiede and Kang 2011).

Apart from epithelial compartments, stroma consists of a major part of the gland and is comprised of adipocytes, fibroblasts, immune, lymphatic and vascular cells which support the function and the development of the gland. The stroma, or fat pad, forms the major part of adult and non-lactating mammary glands. During pregnancy and lactation, fat content will be reduced and used for metabolic purpose of milk production. Adipocytes serve as the endocrine reservoir for the mammary epithelium, governing its growth and function (Bartley, Emerman et al. 1981, Maller, Martinson et al. 2010, Inman, Robertson et al. 2015).

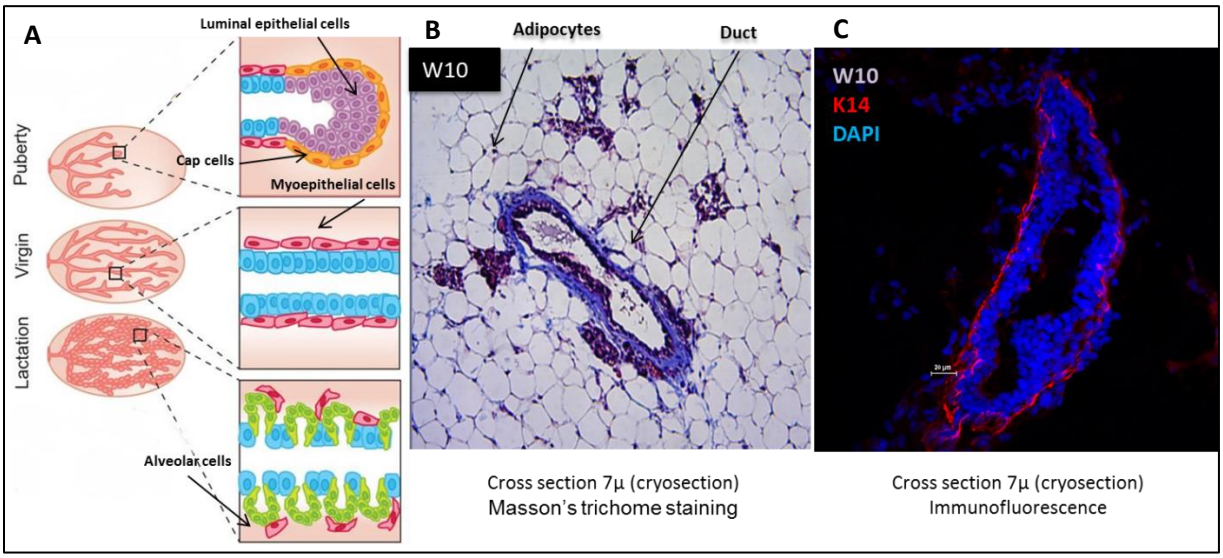

Figure 4) Mammary Gland structure. A) Schematic view of mammary glands structure (Inman, Robertson et al. 2015). B) Masson's trichrome and C) Immunofluorescence staining show a duct consists of the luminal cells surrounded by myoepithelial cells around lumen (Dianati, E. unpublished results).

The epithelium is separated from the stroma by a basement membrane, which consists of two layers; the basal lamina directly underlying the epithelial cells and the reticular lamina underlying connective tissues. The basal lamina provides the physical support and stiffness which are required for overall structure of the gland. It is mainly composed of proteoglycans, collagen IV, growth factors, laminin, fibronectin and cytokines which can bind to fibrillar ECM proteins and basal lamina via polysaccharides named Glycosaminoglycan. ECM and epithelial compartments have dynamic,

reciprocal interactions: cells generate and rebuild the ECM, while ECM dictate and instruct cells (Maller, Martinson et al. 2010, Inman, Robertson et al. 2015).

Fibroblasts, which are embedded in stroma, secrete the growth factors and cytokines and thereby play an important role in survival of epithelial cells and morphogenesis of fat pad. Moreover, fibroblasts are responsible for synthesis of ECM components such as collagens, proteoglycans and fibronectin as well as enzymes, such MMPs, responsible of ECM degradation (Maller, Martinson et al. 2010, Inman, Robertson et al. 2015).

In multicellular organisms and within different organs, communications and interactions between cells are necessary in order to ensure tissues' health and proper function.

1.3 Cell-cell interaction via Intercellular Junctions in the mammary glands

Remodeling of cell-cell contact is a requisite for proper development of mammary glands, both in responding and dictating increased cell proliferation in puberty and pregnancy, cell differentiation in lactation or increased apoptosis and cellular remodeling during involution. Loss of function in any of the adhesion complexes lead to perturbation of normal tissue function (Dbouk, Mroue et al. 2009) (Liu, Radisky et al. 2005). Proper mammary glands' function and development require constant interaction between stroma and epithelium and also within the epithelium between the luminal and myoepithelial cells (Sternlicht, Kouros-Mehr et al. 2006). Epithelial cell-cell contact is provided by means of intercellular junctions, namely gap, tight and adherens junctions (Figure 5). They provide direct cell-cell and cell-ECM communications as well as structural support, and promote and initiate signaling cascades (Gumbiner 1996) (Dbouk, Mroue et al. 2009).

1.3.1 Gap junctions

Gap junctions (GJs) are specialized class of channels composed of Connexins (Cxs) providing direct cell-cell communication by connecting the cytoplasm of two adjacent cells. GJs allow the passage of ions and macromolecules such as amino acids, second messengers and metabolites less than 1kDa via passive diffusion (Evans and Martin 2002) (Goodenough, Goliger et al. 1996) (Herve, Bourmeyster et al. 2007). GJs are essential in embryonic growth and development as well as tissue differentiation (El-Sabban, Abi-Mosleh et al. 2003) (Fentiman, Hurst et al. 1979) (Warner 1992). GJs between epithelial cells of mice mammary glands were first detected by electron microscopy (Pitelka, Hamamoto et al. 1973). The presence of functional GJs in the lobules of lactating gland was confirmed by injection of

Lucifer yellow, a dye that can pass though GJs (Pitelka, Hamamoto et al. 1973). Interaction between myoepithelial and luminal cells in the mammary gland was suggested for the first time in 1997 (Radice, Ferreira-Cornwell et al. 1997).

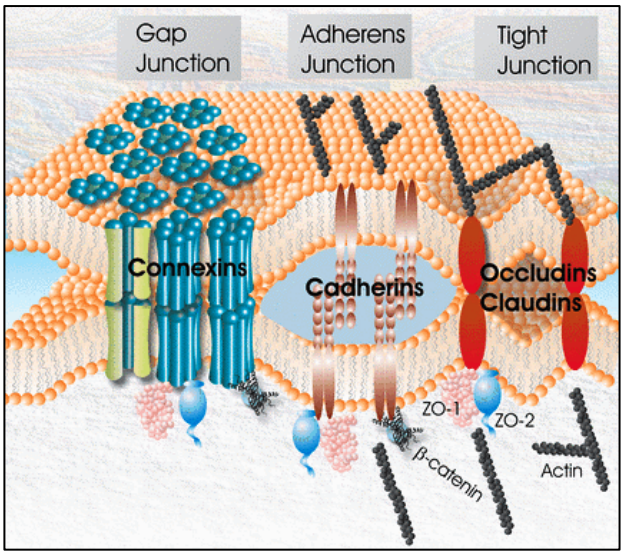

Figure 5) Intercellular junctions. Junctional complexes arranged in a nexus (Laird 2006).

Twenty and twenty-one different Cxs genes are expressed in mice and human, respectively (Oyamada, Oyamada et al. 2005) (Sohl and Willecke 2003). Only four, Cxs, Cx43, Cx26, Cx30 and Cx32, are expressed in mice mammary gland while just two of them, Cx43 and Cx26, are expressed in the human breast (McLachlan, Shao et al. 2007) (Figure 6). Cx26, Cx32 and Cx30 are expressed in luminal epithelial cells, and their expression varies with stages of development. Cx26 is expressed from mid-gestation and through lactation, Cx32 after parturition and across lactation and Cx30 from mid-gestation and during lactation (Locke, Stein et al. 2004) (Monaghan, Perusinghe et al. 1994) (Locke, Jamieson et al. 2007) (Talhouk, Elble et al. 2005) (McLachlan, Shao et al. 2007). It has been suggested that these Cxs can make channels composed of one type or various types of connexins (homomeric and heteromeric channels) during the lactation when they are all expressed to support the milk production (Locke, Stein et al. 2004, Talhouk, Elble et al. 2005). On the other hand, Cx43 is constantly expressed between myoepithelial cells and in fibroblasts of the stroma (Talhouk, Elble et al. 2005), whereas its expression between myoepithelial and luminal cells is under debate.

To address the role of Cxs in glands function, various knockout and ectopic transgenic mice models were created. Cx32 and Cx30 knockouts are fertile and exhibited no lactation abnormalities (Teubner, Michel et al. 2003) (Nelles, Butzler et al. 1996). Cx26 knockouts are embryonically lethal (Gabriel, Jung et al. 1998) and Cx43 knockout mice die within few hours after birth (Reaume, de Sousa et al. 1995). Though, Cxs conditional knockouts in mammary glands of mice have provided more information for the field. Cx26 ectopic ablation before puberty has resulted in abrogated lobo-alveolar development and function at the time of lactation, whereas this ablation during mid or late pregnancy has shown no alteration (Stewart, Plante et al. 2014) (Bry, Maass et al. 2004). A mouse model of the human disease oculodentodigital dysplasia, which result from mutations within the gene encoding for Cx43, has been used to investigate the role of Cx43 in mammary gland development (Flenniken, Osborne et al. 2005) (Plante and Laird 2008). These mice, named *Gja1*$^{Jrt/+}$, harbor a dominant-negative point mutation, resulting in a decrease of approximatively 90% of Cx43 protein levels (Flenniken, Osborne et al. 2005) (Plante and Laird 2008). Cx43 reduction resulted in reduced GJIC in myoepithelial cells, as well as in a delayed ductal development and defective lactation (Plante and Laird 2008).

Connexins gene expression, protein level and protein function play a role in growth, differentiation and developmental signaling (Dbouk, Mroue et al. 2009). Connexins (Cxs) polypeptide contains two extracellular loops (E1 and E2), four transmembrane domains (M1-M4), a short cytoplasmic amino-terminal domain (N), a carboxyl-terminal cytoplasmic domain (C) and one cytoplasmic loop between M2 and M3 (El-Sabban, Abi-Mosleh et al. 2003) (Figure 6). Sequence comparison established a high conservation between the different Cxs, the most divergent domains being the cytoplasmic loop and the carboxyl terminal. These differences are thought to be responsible for specific functional properties of the different Cxs (Dbouk, Mroue et al. 2009)(Locke and Harris 2009).

Six Cxs within one cell assemble together to form a connexon (or hemichannel) (Figure 6). Hemichannels can be homomeric, i.e. formed by six identical Cxs, or heteromeric, formed by at least two or more different types of Cxs. Two connexons from adjacent cells dock together and make a GJs channel which can also be homotypic (identical connexons) or heterotypic (different connexons). The permeability, gating, functionality and conductance of GJs channels may vary between homomeric/heteromeric hemichannels as well as homotypic/heterotypic channels, based on Cxs content (Cottrell and Burt 2001) (El-Sabban, Abi-Mosleh et al. 2003). 100 to 1000 GJs channels cluster together and form GJs plaques at the cell membrane.

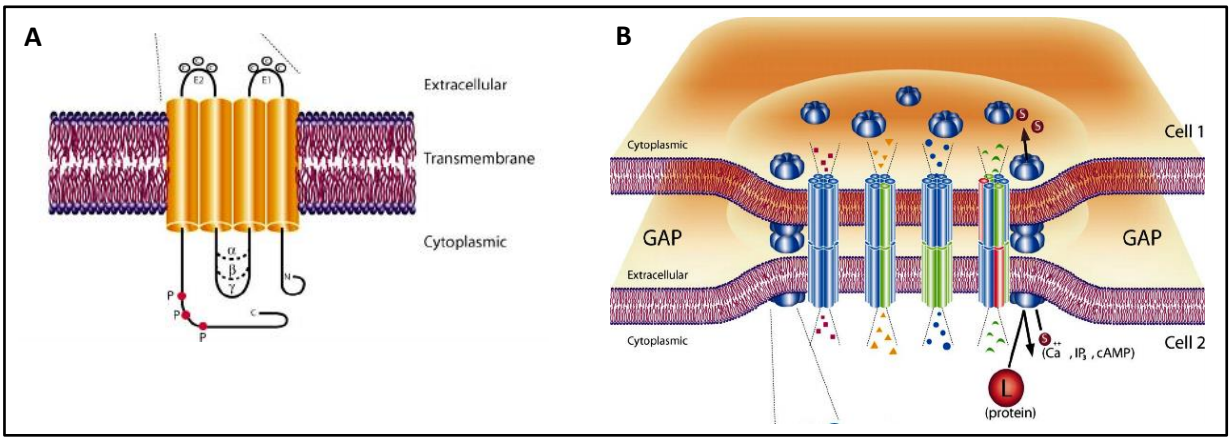

Figure 6) Connexins structure and gap junction channels. A. Structure of Cx polypeptide. B. Cxs, connexons, gap junction channel (El-Sabban, Abi-Mosleh et al. 2003).

Like other integral membrane proteins Cxs form in ER (Figure 7). Cxs mRNA will be translated to the polypeptide by ribosomes attached to the rough endoplasmic reticulum (ER) (Hurtley and Helenius 1989) (Dbouk, Mroue et al. 2009) (Falk, Buehler et al. 1997) (Lampe and Lau 2000). Most Cxs oligomerization into connexons is thought to happen in the in ER, but some Cxs stay in monomer until they reach the Golgi apparatus; for instance, Cx43 and Cx46 oligomerization occurs in trans-Golgi network (Musil and Goodenough 1993) (Koval, Harley et al. 1997). Upon oligomerization, connexons are packed into vesicles between ER and Golgi and delivered to the membrane. Insertion of connexons in the plasma membrane is mediated by microtubules in regions containing adherens junctions (Shaw, Fay et al. 2007). Another exception for this pathway is Cx26 which upon post-translation moves from ER to the plasma membrane without passing the Golgi network (Ahmad and Evans 2002).

Cxs have a short half-life of few hours (Laird, Puranam et al. 1991) (Fallon and Goodenough 1981). New connexons are recruited to the periphery of existing GJs plaques, while the degradation of old connexons occurs in the middle of the plaque. GJs channels internalization occurs via formation of annular junctions, a unique double-membrane vesicle that contain the entire GJs channel. Following internalization, GJs channel will be disassembled to connexons and consequently to Cxs. Cxs final turnover occurs either by proteasome or lysosome degradation (Dbouk, Mroue et al. 2009).

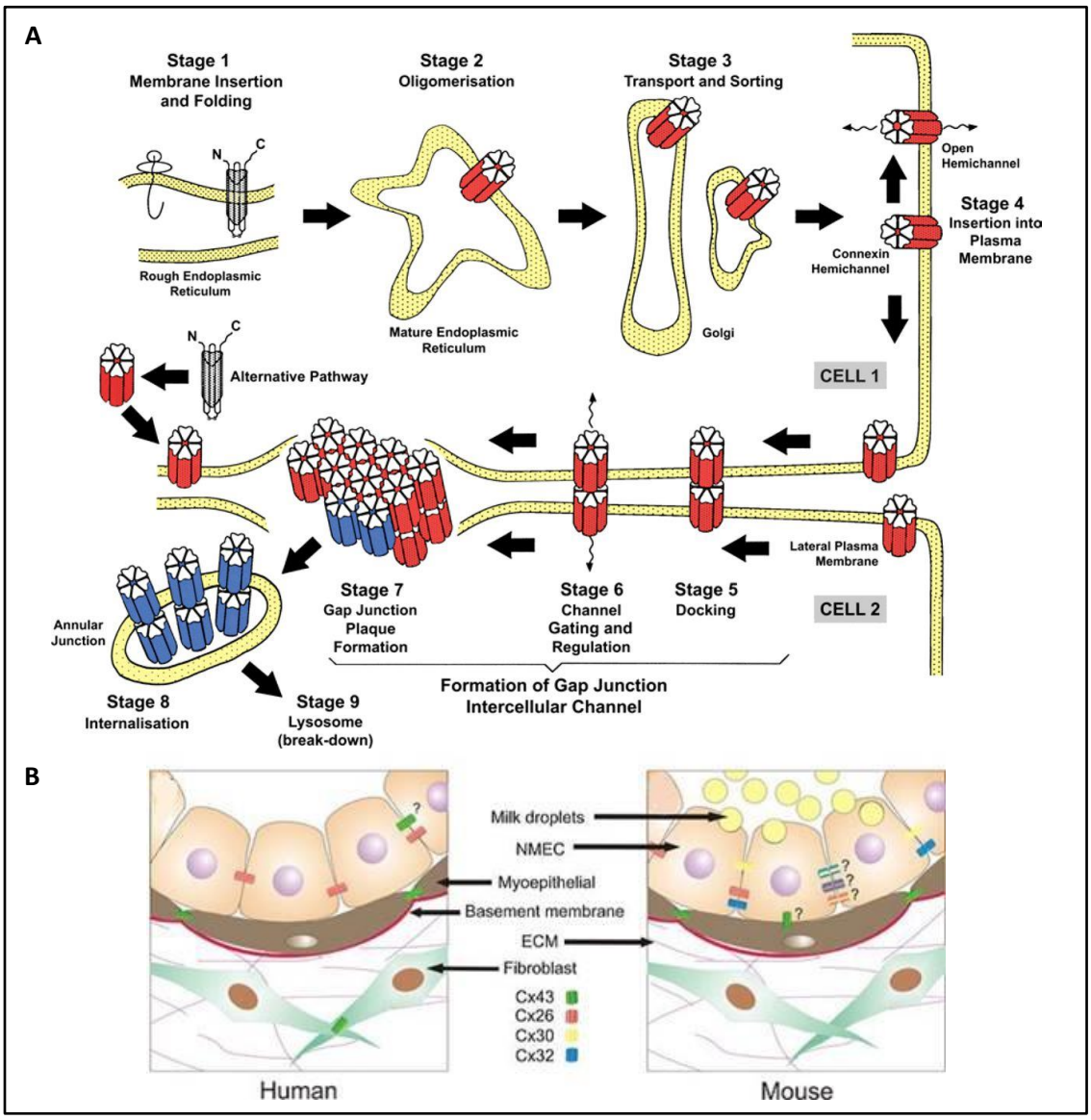

Figure 7) Connexins trafficking and expression in mammary glands. A. Cxs trafficking, protein synthesis and trafficking to the membrane (Evans, De Vuyst et al. 2006). B. Cxs which are expressed in human breast and mice mammary glands (McLachlan, Shao et al. 2007).

Various physiological aspects such as, PH, voltage, calcium abundance, type of Cxs composing the channel as well as Cxs phosphorylation are implicated in channel conductance and permeability, trafficking, assembly and disassembly of GJs (Dbouk, Mroue et al. 2009) (Lampe and Lau 2000). The most common post-translational modification is the phosphorylation of Cxs at various residues, mostly present within the C-terminal domains, but also sometimes within the N-terminal or cytoplasmic loop of different Cxs. Protein kinases including v-src and c-src kinases, protein kinase C, MAPK (mitogen-activated protein kinase), cdc2 kinase, casein kinase 1 and protein kinase A (PKA) and phosphatases

interact with members of Cxs family (Solan and Lampe 2005) (Lampe and Lau 2004) (Laird 2005). Phosphorylation can occur during Golgi or plasma membrane trafficking. Some unphosphorylated Cxs have been found in plasma membrane establishing that sometimes phosphorylation can occurs at the plasma membrane (Lampe and Lau 2000). Studies have shown that Cxs phosphorylation is required for their trafficking, assembly and disassembly, degradation and gating of GJs channels. These effects are very specific to the residues of phosphorylation, i.e. phosphorylation of Cx43 by the same kinase at different residues can positively or negatively influence gap junctions communication (El-Sabban, Abi-Mosleh et al. 2003) (Lampe and Lau 2000). Cx26 having a short C-terminal tail, has not been reported to be phosphorylated and doesn't require phosphorylation for formation of functional gap junctions (Lampe and Lau 2000).

1.3.2 Tight junctions

Tight Junctions (TJs) were first identified using electron microscopy (EM) as closely associated areas of two cells whose membranes join together (Farquhar and Palade 1963). In 70th decade using the freeze fracture technique, TJs were observed as parallel strands or gates along epithelial cells interconnected by shorter fibrils which restrict the passage of ions and macromolecules such as proteins and lipids by diffusion (Fromter 1972) (Chalcroft and Bullivant 1970). TJs provide the polarity of epithelial cells by forming a belt-like structure at apical region of basolateral membrane (Tsukita, Furuse et al. 2001). TJs provide a dynamic selective semi-permeable barrier at paracellular apex of epithelial cells and maintain the required tissue hemostasis within glandular tissue, such as the mammary glands (Brennan, Offiah et al. 2010).

Components of TJs are generally categorized in to the 1) Integral membrane proteins, 2) Scaffolding cytoplasmic associated proteins and 3) Signaling proteins.

1) Integral membrane proteins: TJs are composed of a branching network of sealing strands. Each strand is formed from a row of transmembranes proteins embedded in both plasma membranes, with extracellular domains joining one another directly (Figure 8). Occludin, the first molecule identified at TJs (Furuse, Hirase et al. 1993), is a transmembrane protein that consisting of two extracellular loops and a long cytoplasmic tail containing several protein binding domains. Claudins are a family of integral membrane protein that consists of 24 members (Furuse, Sasaki et al. 1998). Claudins bind to PDZ domain of other TJs proteins, including Zona Occludens (ZO)-1, -2 and -3, through their short cytoplasmic tails (Itoh, Furuse et al. 1999). Barrier properties rely on claudins which are responsible of

controlling electrical conductance, size and charge selectivity which form size- and charge-selective aqueous pores (Van Itallie and Anderson 2006).

2) Peripherally associated cytoplasmic or scaffolding proteins: About 30 molecules have been identified in this category, which either contain PDZ domain or not (Mitic and Anderson 1998). PDZ domain proteins ZO-1, -2, and -3 are involved in recruiting signaling proteins of cytoskeleton, actin filaments and clustering TJs membrane integral membrane protein (Fanning, Jameson et al. 1998, Itoh, Furuse et al. 1999) (Wittchen, Haskins et al. 1999). Zona occludens proteins can also can interact with other integral membrane proteins such as Occludin (Fanning, Jameson et al. 1998) (Ding, Cong et al. 2017), claudin 1-8 (Itoh, Furuse et al. 1999) (Van Itallie, Tietgens et al. 2017) and actin (Fanning, Jameson et al. 1998) (Wittchen, Haskins et al. 1999).

3) Signaling proteins: These proteins may be involved in junction assembly, barrier regulation and gene transcription. Protein kinase A, C and heteromeric G-proteins are members of this family. Protein kinase A has been shown to promote epithelial barrier function (Suzuki, Yamanaka et al. 2001, Aljameeli, Thakkar et al. 2017) (Denker, Saha et al. 1996). Protein kinase C and A, affects both assembly and disassembly of TJs, thus regulate the dynamics of TJs formation (Aljameeli, Thakkar et al. 2017) (Mullin, Laughlin et al. 2000) (Jian, Chen et al. 2015).

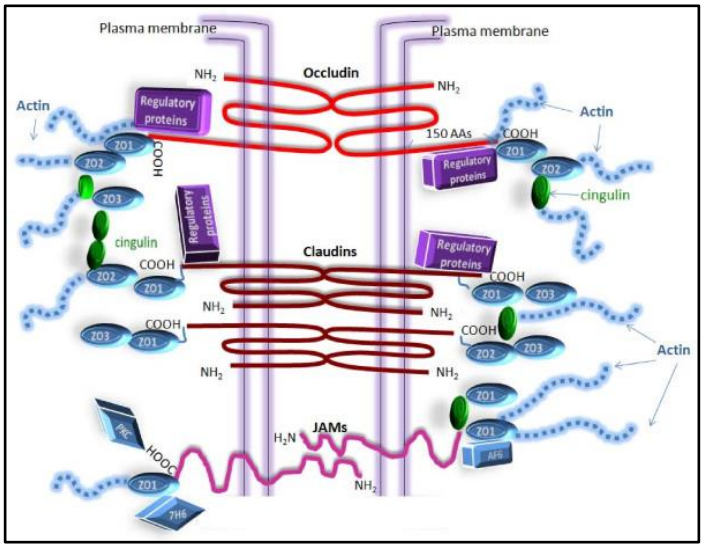

Figure 8) Tight junctions compositions. Schematic representation of tight junctions between two adjacent cells (Redzic 2011).

TJs play a role in the differential permeability needed in the lactating mammary gland, regulating adhesion, migration, polarity and differentiation (Lelievre 2010). Studies in mammary gland, have shown that TJs are influenced by lactogenic hormones such as prolactin and glucocorticoids, as well as progesterone (Nguyen, Parlow et al. 2001) (Stelwagen, McFadden et al. 1999) (Rubenstein, Guan et al. 2003). TJs are essential for prevention of milk leakage during lactation period (Itoh and Bissell 2003). Expression of claudin-1, -3 and -4 has been reported to vary during development while claudin-7 remains constant (Blackman, Russell et al. 2005, Blanchard, Watson et al. 2006). Changes in TJs proteins, occludin and claudins, were associated with alteration of cell polarity, cell cycle progression, proliferation, migration, invasion as well as apoptosis in cancer cells (Osanai, Murata et al. 2006, Osanai, Murata et al. 2007, Osanai, Murata et al. 2007) (Frankel, Aronheim et al. 2005) (Balda, Garrett et al. 2003) (Hoevel, Macek et al. 2004) (Brennan, Offiah et al. 2010) (Lanigan, O'Connor et al. 2007) (Michl, Barth et al. 2003, Mima, Tsutsumi et al. 2005) (Swisshelm, Machl et al. 1999). Although the role of TJs components in breast cancer has been substantially studied, the modulation of their expression as well as their composition within junctional nexus across the mammary gland development is not well studied.

1.3.3 Adherens junctions

Adherens junctions (AJs) are mainly implicated in providing the adhesive contacts between cells and play an important role in tissue and organ architecture during embryogenesis and in adulthood (Gumbiner 1996). They are also essential for intracellular signaling mechanisms (Aberle, Schwartz et al. 1996, Conacci-Sorrell, Zhurinsky et al. 2002), regulation of cell polarity, differentiation, growth and migration (Larue, Antos et al. 1996). The functional units of cell adhesion are multiprotein complexes made by the cell adhesion molecules, the cytoplasmic/peripheral membrane proteins and ECM proteins (Gumbiner 1996). Cadherins form complexes with cytoplasmic proteins called Catenins which make the bridge between Cadherins and the actin cytoskeleton (Kemler 1993) (Figure 9). Dysfunction of the E-cadherin/catenin adhesion complex has also been reported in many human cancers (Behrens 1999, Conacci-Sorrell, Zhurinsky et al. 2002).

Cadherins are transmembrane Ca^{2+} dependent hemophilic adhesion molecules. The classical Cadherins family consists of many members, named after the tissue which they were first identified in: E-cadherin in epithelial cells, N-cadherin in the nervous system, VE-Cadherin in the vascular endothelial and P-cadherin in placenta (Nose and Takeichi 1986) (Rudini and Dejana 2008).

All the classical Cadherins have the same structure: an extracellular domain consists of five ectodomains, EC1 to EC5 which bind to calcium, and a highly conserved cytoplasmic tail including binding site for either β-catenin or γ-catenin (Saito, Tucker et al. 2012). EC domains form a strand dimer which contains two parallel monomers pointed toward the adjacent cells. Each strand dimer interacts with two antiparallel dimers from neighboring cells and together they form a linear ribbon structure (Figure 9).

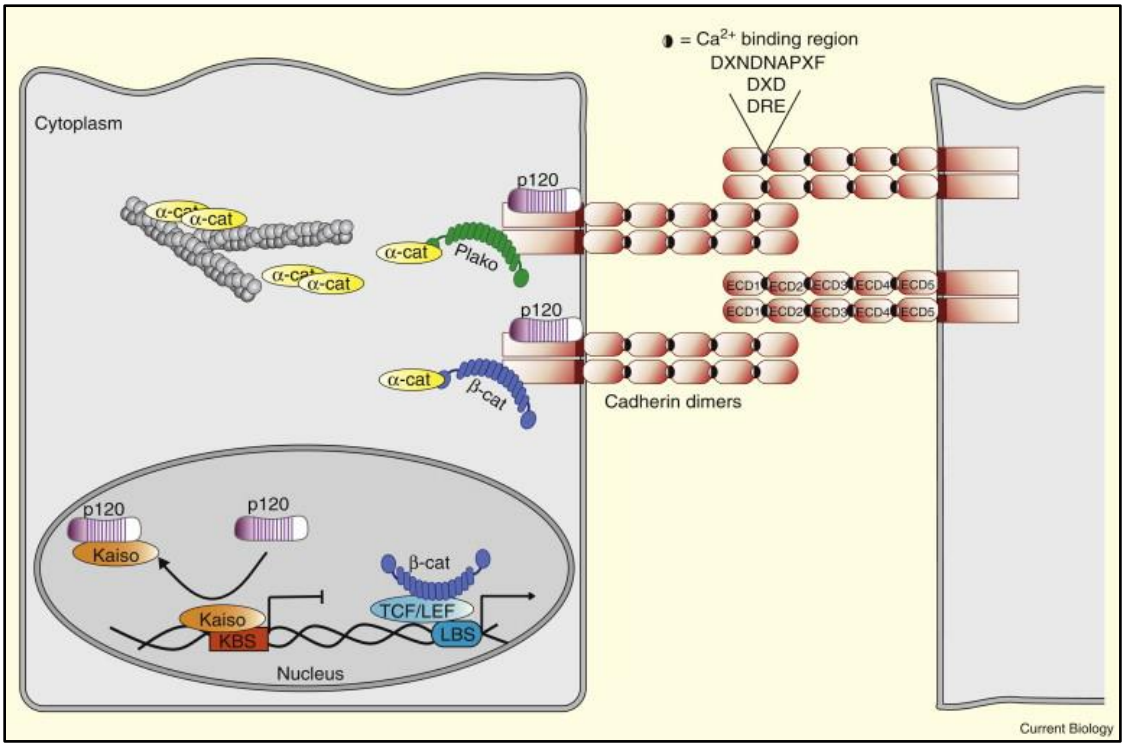

Figure 9) Schematic view of Adherens juctions (AJs). Cadherin-catenin complex organization and function at the cell membrane connected to the actin cytoskeleton. β-catenin and p120ctn, when released from junctions and free in the cytosol, may translocate to the nucleus and regulate cell transcription (Rudini and Dejana 2008).

Cadherins genes are frequently silenced, mutated or dysregulated in breast cancer (Andrews, Kim et al. 2012). Cadherins switching, a hallmark of the epithelial to mesenchymal transition (EMT), is very common in cancer, and leads to increased motility, invasiveness and proliferation of metastatic cancer cells (Andrews, Kim et al. 2012). Type I cadherins include E-, N-, P-, R-cadherins are all expressed in the mammary glands (Andrews, Kim et al. 2012). E-cadherin, the best characterized member of the

Cadherin family, is required for alvelologenesis in lactating mouse mammary glands (Boussadia, Kutsch et al. 2002) and is abundantly expressed on the lateral membranes of TEBs body cells as well as all of mammary epithelial cells (Andrews, Kim et al. 2012). E-cadherin's residence in adherens junctions enables its cytoplasmic tail binding to p120, γ-catenin and β-catenin, facilitating its connection to the actin cytoskeleton (Andrews, Kim et al. 2012). E-cadherin loss has been linked to increase EMT, motility, invasiveness and overall metastasis in breast cancer (Birchmeier 1995) (Gamallo, Palacios et al. 1993, Moll, Mitze et al. 1993, Palacios, Benito et al. 1995). N-cadherin is majorly expressed in stroma and its mis-expression and localization in epithelial cells has been associated with breast cancer (Andrews, Kim et al. 2012). P-cadherin is mostly distributed in basally located cap cells and the basal cell layer of stratified epithelia in adult epithelial tissues (Radice, Sauer et al. 2003) (Nose and Takeichi 1986, Shimoyama and Hirohashi 1991, Hines, Jin et al. 1999). Little is known about the function, regulation and differential expression of P-cadherin in the mammary glands, but its aberrant expression has been established as a poor prognostic factor patient survival in breast cancer (Paredes, Albergaria et al. 2005) (Palacios, Benito et al. 1995, Peralta Soler, Knudsen et al. 1999, Gamallo, Moreno-Bueno et al. 2001, Paredes, Milanezi et al. 2002, Paredes, Milanezi et al. 2002, Kovacs, Dhillon et al. 2003, Kovacs and Walker 2003). Virgin P-cadherin null female mice exhibited premature differentiation of the mammary gland, resulted in hyperplasia and dysplasia with age (Radice, Ferreira-Cornwell et al. 1997).

β-catenin is a cytoplasmic component of AJs that binds to the cytoplasmic tail of Cadherins, but is also involved in signaling, acting as a transcription factor of the canonical Wnt pathway. In normal breast, the majority of β-catenin resides in AJs at cell membrane, while a minor portion is involved in signaling (Incassati, Chandramouli et al. 2010). E-cadherin/β-catenin adhesion complex inhibits β-catenin's function as a transcription factor. Free cytosolic β-catenin, i.e. not bound to Cadherin, will be either degraded through destruction complex and serine/threonine phosphorylation in non-canonical pathway, or accumulated in cytosol and translocated into the nucleus after activation of Wnt/frizzled (Figure 10A). Once in the nucleus, β-catenin associates with members of the T-cell factor/lymphoid-enhanced factor (TCF/LEF) family of transcription factors and regulates the expression of target genes (Figure 10B) (Incassati, Chandramouli et al. 2010) (Rudini and Dejana 2008). As a result, the relative amount of cadherin-bound β-catenin and free β-catenin is critical to keep cellular integrity, cytoskeletal dynamic and tissue homeostasis between different cell types within a tissue (Andrews, Kim et al. 2012) (Perez-Moreno and Fuchs 2006). Phosphorylation at Ser33, Ser37, and Thr41 by GSK3-β and at Ser45 by priming kinase CKια, are required for β-catenin's recognition by proteasome, its ubiquitination and

degradation (Liu et al., 2002). It is also known that phosphorylation of β-catenin at Ser675 prevents it from degradation and thereby promote its transcriptional activity (Spirli, Locatelli et al. 2013) (Taurin, Sandbo et al. 2006). Another phosphorylation site at Tyr654 is thought to be implicated in the association of β-catenin with E-cadherin in cell-cell adhesion (Roura, Miravet et al. 1999).

β-catenin signaling is required for bud and placode development during embryogenesis, ductal branching at puberty and in cycling adult, lobuloalveolar development throughout pregnancy and nursing the pups during lactation (Tepera, McCrea et al. 2003) (Incassati, Chandramouli et al. 2010). Augmentation of β-Catenin levels in the cytoplasm and nucleus is observed in primary breast cancers and correlated with worse patient outcome (Lin, Xia et al. 2000) (Ozaki, Ikeda et al. 2005).

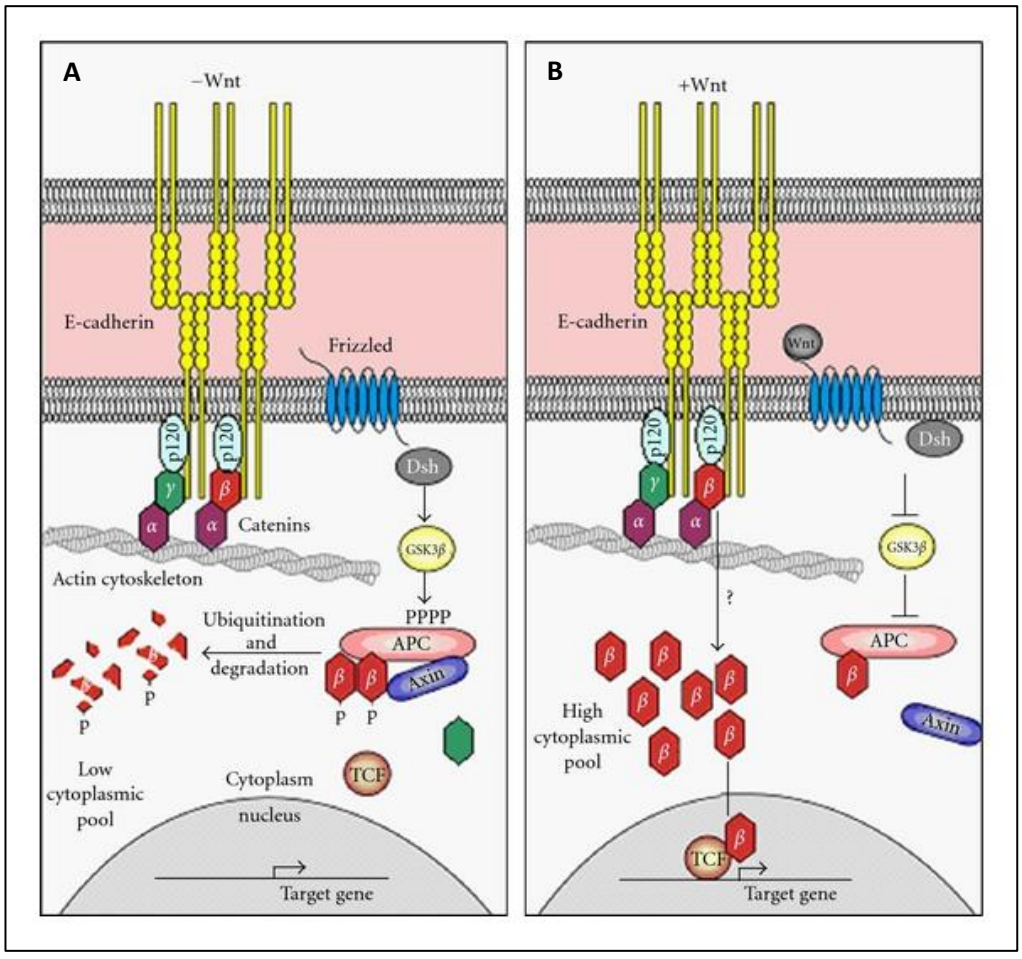

Figure 10) Dual function of β-catenin. A) In absence of wnt ligand, non-membranous β-catenin resides in a complex with APC and Axin and gets phosphorylated and be targeted for ubiquitination. **B)** When wnt ligand is active, β-catenin gets dissociated from destruction complex, accumulate in cytoplasm and get translocated to the nucleus (Tian, Liu et al. 2011).

P120Catenin (p120ctn) binds to Cadherins at the cytoplasmic tail and is critical for the surface stability of cadherin-catenin cell adhesion complexes. Similar to β-catenin, Wnt activation can dissociate p120ctn from membrane and translocate and stabilize it in the nucleus (Rudini and Dejana 2008) (Ishiyama, Lee et al. 2010).

Finally, α-catenin is the dynamic regulator of the actin cytoskeleton, linking Cadherins to the actin cytoskeleton. For a long time, α-catenin has been considered to be the attachment molecule of AJs using indirect β-catenin and F-actin interaction. However, the exact mechanism is still unknown, since several actin-binding proteins present at AJs, such as Eplin (Epithelial protein lost in neoplasm), vinculin, formin-1, α-catenin and α–actin, all influence the actin cytoskeleton indirectly (Rudini and Dejana 2008).

In brief, proper regulation and expression of AJs components have been shown to be crucial for mammary gland development and differentiation. They are also considered as key regulators of the junctional nexus by facilitating the anchoring of other junctions, including GJs.

1.3.4 Junctional proteins interplay with each other in different tissues

Previous studies have shown that GJs' proteins, Cxs, play an active role in intercellular signaling. The interaction of GJs proteins with components of AJs and TJs and cytoskeletal elements has clearly established the interplay between these adhesion complexes (Kojima, Kokai et al. 2001) (Herve, Bourmeyster et al. 2007) (Herve, Bourmeyster et al. 2004) (Dbouk, Mroue et al. 2009) (Talhouk, Mroue et al. 2008). Cxs attachment to the other interacting proteins is prominent for GJs stability, assembly and function (Herve, Bourmeyster et al. 2007) (Jongen, Fitzgerald et al. 1991, Herve, Bourmeyster et al. 2004). There are many examples supporting the crucial role of junctional proteins interplay. For instance, microtubules-Cxs and microtubules-Cadherins interaction allows direct transport of Cxs to the plasma membrane and delivery of new connexons to pre-existing sites of AJs, respectively (Shaw, Fay et al. 2007). Similarly, it has been demonstrated that E-Cadherin first binds to actin filaments, and Cx43 can then be transported to the cell membrane in skin cells (Hernandez-Blazque et al, 2001). β-catenin Interaction with Cx43 keeps it at the cell membrane in liver cells (Ale Agha N, 2009). Over-expression of Cx43 significantly induced E-cadherin expression in lung cancer cells (Hong-Tao et al, 2008). A positive correlation has been observed between E-cadherin, β-catenin and Cx43 expression in colorectal cancer (Kanczuga-Koda et al, 2014). It has been shown that heterocellular interactions between mammary gland cells SCg6 (myoepithelial-like cells) and SCp2 (epithelial-like cells) cause an increased association

between Cxs (Cx32, Cx43 and Cx30) on one hand, and between α-catenin and ZO-2 on the other hand, and result in the recruitment of β-catenin into GJs complex, thus preventing its translocation to the nucleus (Talhouk, Mroue et al. 2008). Finally, interactions between E-cadherin and Cx43 in mouse epidermal cells (Jongen, Fitzgerald et al. 1991), as well as between β-catenin and Cx43 in cardiomyocytes and hepatocytes (Ai, Fischer et al. 2000 & Fujimoto et al, 1997) all demonstrated that junctional proteins do interplay in various tissues. However, little is known about the role and the dynamic of these interactions in mammary glands.

Post-natal mammary gland development like many other endocrine dependent-organs is orchestrated by the hormones secreted under the regulation of hypothalamus and pituitary axis.

1.4 Endocrine system and hormones hormones in mammary gland development

1.4.1 Endocrine system

The endocrine system is comprised of multiple peripheral endocrine glands: ovary, testis, thyroid and adrenal glands which are governed by the hypothalamus-pituitary axis (Figure 11A). The hypothalamus of the brain secretes releasing hormones (RHs) that act on the pituitary gland and cause the release of stimulating hormones (SHs) (Figure 11A) which in turn control peripheral endocrine organs. In addition, posterior pituitary receiving the axonal projection from hypothalamus is in charge of vasopressin and oxytocin release.

Anterior pituitary is also responsible for secretion of Growth hormone (GH) and Prolactin (PRL). Moreover, there is a negative loop feedback between hormones released by peripheral endocrine glands talking back to both pituitary and hypothalamus. These multiple interactions and feedback loops ensure hemostasis of the system (Brisken and Ataca 2015).

1.4.2 Role of hormones and their receptors in mammary gland development

While embryonic development is hormonal independent and regulated by epithelial-mesenchymal cues, pubertal and reproductive stages are tightly orchestrated by ovarian and pituitary hormones as well as growth factors (Gumbiner 1996) (Figure 11B, Figure 12). Mammary branching is

comprised of pubertal ductal and secondary branching, as well as adult tertiary branching, each one differently regulated. Ovarian and pituitary hormones are absolutely essential for pubertal ductal mammary development (Figure 11). Ductal branching requires growth hormone (GH), estrogen (E2) and estrogen receptor α (ERα). Pituitary growth hormone, which is already present before the pubertal surge of ovarian estrogens, acts via its receptor on mammary stroma cells to elicit the expression of stromal IGF-1 which itself is required for TEBs formation and epithelial branching. During puberty, estrogen levels increase and induce progesterone expression (Haslam and Shyamala 1981). Progesterone induces dichotomous branching till the mid-pregnancy; further alveologenesis require epithelial prolactin signaling (Brisken 2002) (Figure 11B). Likewise, tertiary side-branching in the cycling adult requires progesterone (P4) and its receptor (PR).

Estrogen Receptor α; 17β-estradiol and other estrogens metabolites are essential for proper function of the female reproductive organs and can activate ERα and β. Exogenous estrogen can rescue mammary development in ovariectomized mice, thus suggesting that it acts as an essential "on-switch" (Daniel, Silberstein et al. 1987, Sternlicht 2006). Many years ago, using xenograft mouse models, it has been shown that epithelial steroids are not necessary and sufficient for epithelial proliferation and is mostly regulated through paracrine factors by stromal hormones (Cunha, Young et al. 1997). Subsequent research suggested that ERα was required in both the epithelium and the stroma for proper mammary gland development (Mueller, Clark et al. 2002). More recently, it has been established that the proper development of the mammary gland requires the expression of estrogen receptor (ERα) in epithelium, but not at the stroma (Mallepell, Krust et al. 2006). ERα is crucial for ductal elongation during puberty as well as growth and maintenance of alveolar cells during pregnancy. The role of ERβ is not well defined in the mammary glands. Estrogen can also induce progesterone expression via PR inducible effect (Haslam and Shyamala 1981). ERα is critical for estrogen to act on hypothalamus-hypophysis axis to induce LH and FSH and acts on the pituitary gland to stimulate prolactin synthesis and secretion (Scully, Gleiberman et al. 1997) and in absence of ERα, estrogen could not induce PR (Hewitt and Korach 2000) (Figure 11B, 12). While estrogen alone can cause ductal branching, progesterone requires estrogen to from mature alveoli (Ruan, Monaco et al. 2005).

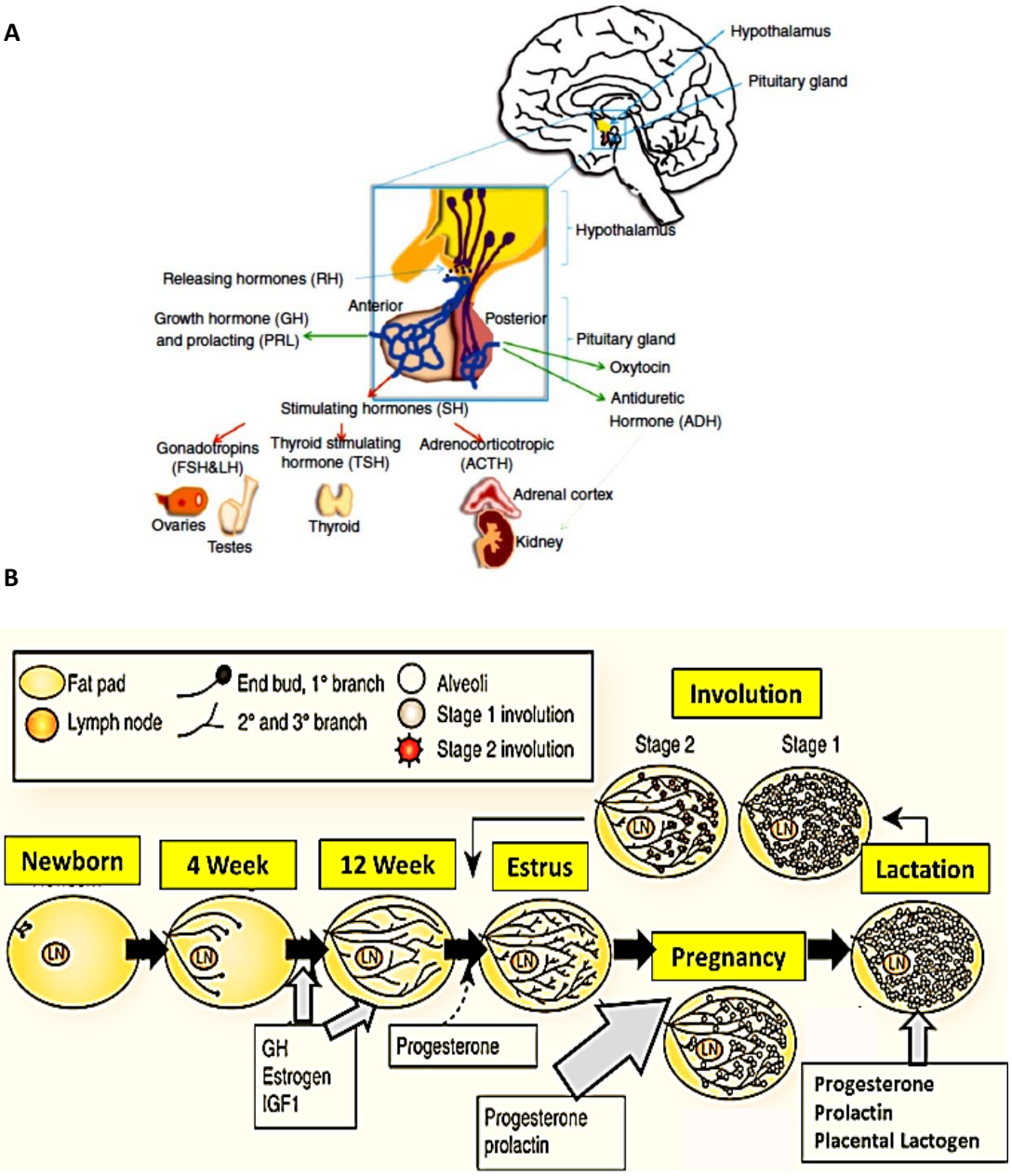

Figure 11) Endocrine system and different hormones involved during mammary gland development. A. Schematic view of hypothalamus-pituitary axis and the peripheral endocrine glands (Brisken and Ataca 2015). B. Schematic view represents that post-natal mammary gland development is tightly orchestrated by hormones; Estrogen (E2), Progesterone (P4), Prolactin (PRL) and Placental Lactogen (PL). At involution, the mammary gland returns to its pre-pregnancy state through epithelial cell apoptosis and stromal remodeling (Macias and Hinck 2012).

Progesterone receptor (PR); The PR is expressed in both epithelial and stromal compartments in the mouse mammary gland (Haslam and Shyamala 1981, Haslam 1989). It has two isoforms, the longer PR-B and the shorter PR-A. Research using knock-out mice established that PR-B is the crucial isoform in

the mammary glands. In addition using a xenograft model, it has been shown that PR-B expression in epithelium, but not stroma, is a requisite for side branching and alveologenesis (Figure 11B, 12) (Lydon, DeMayo et al. 1995, Brisken, Park et al. 1998) (Brisken and Ataca 2015).

Prolactin Receptor (PRLR); PRLR is expressed in epithelial cells, but not in the stroma (Figure 11B, 12). It is also expressed in B-, T-Lymphocytes and macrophages which then can respond to PRL (Brisken and Ataca 2015). PRLR is required for alveologenesis and milk secretion, but not for ductal outgrowth and side branching (Brisken, Kaur et al. 1999) (Humphreys, Lydon et al. 1997, Brisken, Park et al. 1998). Combinatory effect of progesterone, prolactin and placental lactogen (PL) has pivotal role in differentiation of alveolar structure for synthesis and secretion of the milk (Bachelot and Binart 2007) (Figure 11, Figure 12). Expression of specific differentiation markers, such as milk proteins β-casein and whey acidic proteins (WAP), is lost in PRLR-/- mice (Bachelot and Binart 2007).
While PRL upregulates PR mRNA in the mammary gland (Ormandy et al., 1997) and activates ERα in T47D cells (Gonzalez et al., 2009), PRLR mRNA is induced by progesterone in T47D cells (Tseng and Zhu, 1998) and by estrogen in MCF7 cells (Dong et al., 2006). Progesterone induces expression of the PRLR through tethering to the activated transcription factors C/EBP (CCATT/Enhancer Binding Protein) and Sp1 (Specificity Protein 1) (Goldhar, Duan et al. 2011).

The major mediator of PRLR signaling is ELF-5 and JAk2-STAT5 signaling pathway which are necessary for formation of luminal progenitor cells and establishing the alveologenesis (Liu, Robinson et al. 1997) (Choi, Chakrabarti et al. 2009). In contrast, an important mediator of involution is STAT3 which antagonize STAT5 signaling after weaning and increase the pro-apoptotic pathway (Desrivieres, Kunz et al. 2006)

Growth Hormone (GH); The GH released by the pituitary gland is an important global regulator of mammary gland development and its effect is mainly mediated through secretion of a paracrine factor, IGF-1, from the liver in to the blood (Figure 11B, 12) (Gallego, Binart et al. 2001) (Kleinberg, Feldman et al. 2000) (Brisken and Ataca 2015). IGF-1 role in mammary gland development is discussed in paracrine factor section, below. GH receptor is required in stroma (Gallego, Binart et al. 2001) and IGF-1 is required in epithelium and its local expression is far more important than its systemic level (Gallego, Binart et al. 2001) (Figure 11, Figure 12).

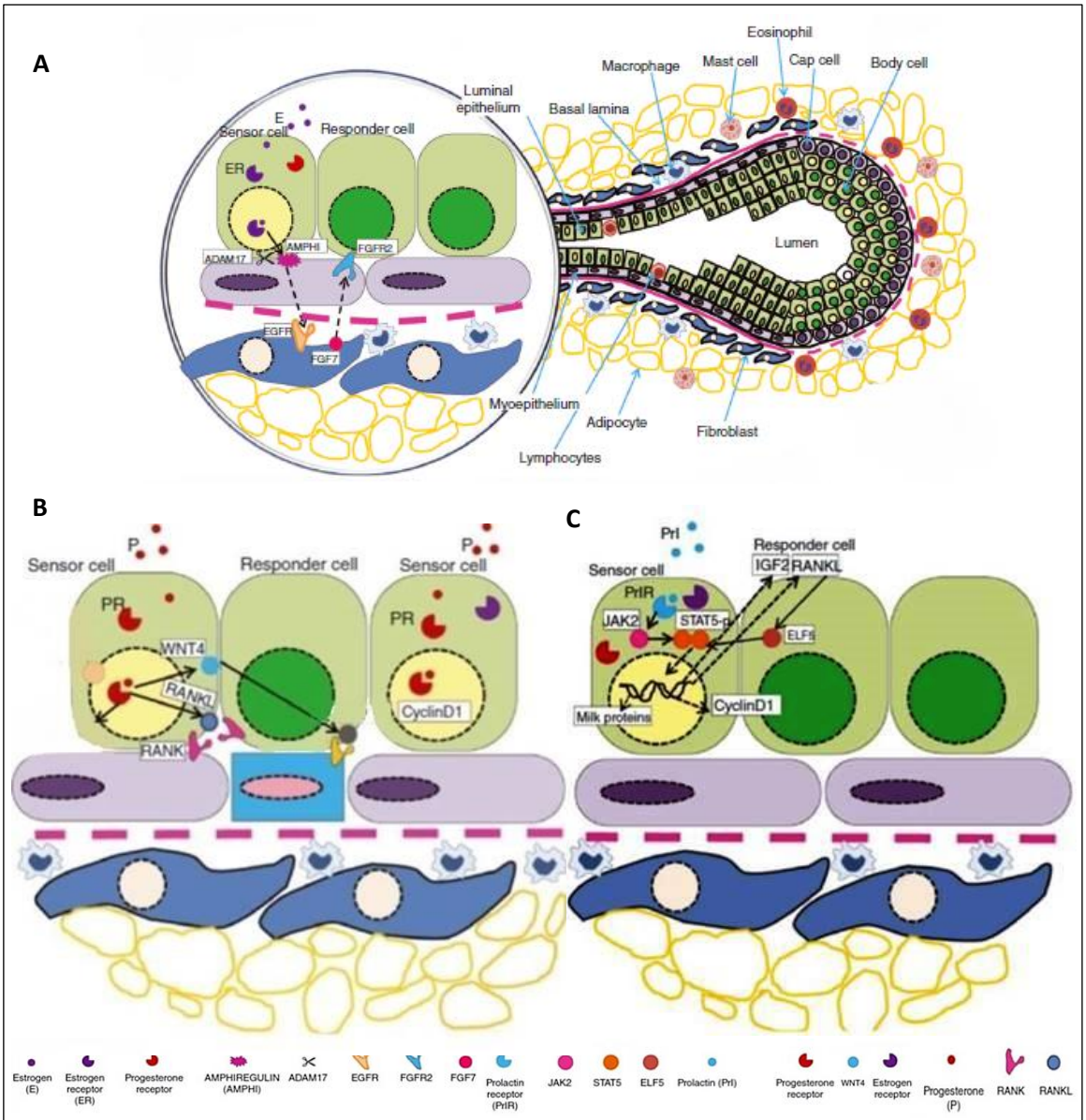

Figure 12) Hormones and paracrine factors requirement for mammary gland function. Hormones and paracrine signals involved in mammary gland development (Brisken and Ataca 2015). **A)** The composition of mammary gland ducts and the signaling required in ductal branching in mammary gland. **B)** In adult tissue, PR and its downstream signalling are involved in mammary gland proper function. **C)** PRL and its signaling to produce milk proteins during alveologenesis.

Glucocorticoid Receptor (GR); GR depletion in epithelium led to delayed alveologenesis, GR is contributing to the cell proliferation during alveologenesis but is not requisite for differentiation of alveoli and milk proteins (Wintermantel, Bock et al. 2005, Brisken and Ataca 2015).

Androgen Receptor (AR); AR depletion has affected TEBs, ductal branching and alveologenesis demonstrating that AR is substantially required for mammary gland development as ductal branching and adult tertiary branching are delayed in AR mutants. Since AR ablation was concomitant with reduction of IGF-I as well as ER which are also essential for normal development, the observed anomalies can't be only associated to AR depletion (Yeh, Hu et al. 2003) (Brisken and Ataca 2015).

Thyroid Hormone; Thyroid hormones are not necessary for ductal outgrowth, but participate in lobulo-alveolar development (Borellini and Oka 1989) (Brisken and Ataca 2015). By activating their receptors, thyroid hormones are involved in accelerating the mammary gland response to the prolactin (Vonderhaar and Greco 1979) (Borellini and Oka 1989).

Local signaling pathways; Growth factors are also vital for gland function and development, providing the cross-talk between stroma and epithelium. Previous observations established that the mammary epithelial cells which are negative for hormone receptors, ERα and PR, can also proliferate had suggested the involvement of paracrine factors. While estradiol and progesterone directly act on epithelial cells, these cells will consequently release paracrine signals that allow other nearby epithelial cells, both luminal and myoepithelial, to proliferate as well (Mallepell, Krust et al. 2006) (Brisken, Park et al. 1998).

EGFR (epidermal growth factor receptor); EGFR promotes mammary branching downstream of ERα. Its ligand, Amphiregulin, is required for 17β-estradiol induced cell proliferation in puberty and ductal outgrowth (Sternlicht, Kouros-Mehr et al. 2006, Ciarloni, Mallepell et al. 2007) (Brisken and Ataca 2015). Transplant experiments showed that stromal, but not epithelial, EGFR expression is required for ductal outgrowth (Wiesen, Young et al. 1999) (Figure 12).

Fibroblast growth factor (FGF) and its receptor (FGFR); FGF and FGFR are also a requisite for proliferation at TEBs (Sternlicht, Kouros-Mehr et al. 2006) (Brisken and Ataca 2015). FGFR2 is required for ductal elongation in the epithelial compartments (Lu, Ewald et al. 2008) (Figure 12).

Tumor growth factor-β (TGFβ); is a negative regulator of mammary branching which limits the epithelial cells proliferation and promotes ECM production (Sternlicht, Kouros-Mehr et al. 2006) (Daniel, Robinson et al. 1996, Ewan, Shyamala et al. 2002).

Insulin-like growth factor (IGF): IGF-1 interacts in a paracrine manner with its receptor on mammary epithelial cells to stimulate TEBs formation and epithelial branching (Sternlicht, Kouros-Mehr et al. 2006). IGF-1 acts downstream of GH or estrogen, since IGF-1 rescue ductal development in hypophysectomized (GH-deficient) animals. In addition, excess GH and estrogen fail to rescue the development of IGF1-null glands (Richards, Klotz et al. 2004). Mammary gland branching morphogenesis is diminished in mice with deficiency of IGF-I. However, it stayed normal upon liver-specific deletion of IGF-I, suggesting that paracrine, but not endocrine, IGF-I is important for mammary branching morphogenesis (Richards, Klotz et al. 2004). IGF-II has also been suggested to be an important mediator of PrlR signaling (Brisken, Ayyannan et al. 2002, Hovey, Harris et al. 2003) (Figure 12).

Receptor activator of nuclear factor kappa-B ligand (RANKL) is also a downstream signaling mediator of PR functions. RANKL ectopic expression can rescue PR^- phenotype (Beleut, Rajaram et al. 2010, Brisken and Ataca 2015) (Fata, Ho et al. 2000, Conneely, Jericevic et al. 2003). Wnt-4 is also required for tertiary side-branching. P4 is required for Wnt-4 expression during pregnancy (Brisken, Heineman et al. 2000).

So far, we have explained the importance of cell-cell interaction as well as the roles played by hormones and paracrine factors for proper mammary gland function. In next section, we will provide some review of literature on how intercellular junctions are also regulated by hormones.

1.5 Regulation of junctional proteins by hormones

GJs and Cxs are regulated by many mechanisms (Su and Lau 2014). GJs coupling as well as Cxs expression can thus be easily up- or down-regulated in response to physiological requirements. Many studies have looked at the effects of hormones and growth factors on Cx43. As an example, estrogen up-regulated while progesterone down-regulated Cx43 in the myometrium (Petrocelli and Lye 1993). As a result, change in the estrogen to progesterone ratio promotes a dramatic 5-fold increase in Cx43 levels in uterus just prior to the labor (Risek, Guthrie et al. 1990, Risek, Klier et al. 1995, Risek and Gilula 1996); Cx43 is then rapidly cleared from myometrium following the labor (Hendrix, Mao et al. 1992).

This Cx43 surge was measured at both mRNA and protein levels in the myometrium, suggesting an increased at the transcription level followed by more protein production (Lye, Nicholson et al. 1993, Chow and Lye 1994). Other hormones similarly influenced Cx43; PTH induces a 4-fold increase in the activity of the Cx43 promoter in rat osteosarcoma cell lines (Mitchell, Ou et al. 2001). Triiodo-thyronine (T3) increases transcription of Cx43 in rat liver, but not in the heart (Stock and Sies 2000), and regulates testicular Cx43 expression in brook trout testis (de Montgolfier, Audet et al. 2011). Cx26 and Cx32 also can be regulated by steroid hormones in the endometrial epithelium of rats (Grummer, Chwalisz et al. 1994, Jahn, Classen-Linke et al. 1995, Risek, Klier et al. 1995, Grummer, Traub et al. 1999), as well as in the liver (Plante et al., 2002). Spatial and temporal expression of Cx43 and Cx26 in endometrium of rats during pregnancy suggests the involvement of hormones and paracrine factors in their regulation (Winterhager, Brummer et al. 1988, Winterhager, Stutenkemper et al. 1991, Winterhager, Grummer et al. 1993, Risek, Klier et al. 1995). It has been established that Estradiol 17-β (E2) and progesterone (P4) differentially regulate the expression of Cx26 and Cx43 in the rat endometrium; E2 increased mRNA levels of Cx43 and Cx26 and formation of gap junctions, while P4 decreased mRNA for both Cxs and did not stimulate gap junction formation (Grummer, Chwalisz et al. 1994, Grummer, Traub et al. 1999). Similar results were observed in human endometrium, showing that Cx26 is enhanced in estrogen-dominated phase and decreased in progesterone-dominant phase of menstrual cycle (Jahn, Classen-Linke et al. 1995). In contrast to these findings, progesterone was identified as a positive regulator of the Cxs expression in the uterus and ovaries (Risek, Klier et al. 1995). In a more recent study, a negative correlation between Cx26 and progesterone was identified, since altered expression and localization of Cx26 along with overexpression of progesterone has been identified in endometrial cancer cells (Lesniewicz, Kanczuga-Koda et al. 2009). Among paracrine factors, higher expression of IGF-IR correlated with Cx26 increase in colorectal carcinogenesis cancers which suggest that IGF-1 can regulate Cx26 in colorectal cancer cells (Sulkowski, Kanczuga-Koda et al. 2006).

Similar to GJs, there are some evidences that TJs are also regulated by hormones, and are linked to the regulation of the epithelium permeability in mammary gland during pregnancy and lactation. The mammary epithelium is highly permeable during pregnancy, and epithelial cells in ducts are more permeable than alveoli (Nguyen, Parlow et al. 2001) (Linzell and Peaker 2009) (Pitelka, Hamamoto et al. 1973). Using ovariectomized mice and P4 exposure, it has been shown that P4 withdrawal at the end of pregnancy is the main driver of TJs closure (Nguyen, Parlow et al. 2001). Further studies suggested that glucocorticoid and activation of PRLR through either PRL or placental lactogen are also required for P4 triggered TJs' closure (Nguyen, Parlow et al. 2001). In another study, TJs formation was enhanced via

PRL and glucocorticoid combinatory stimulation in mouse mammary cell lines (Stelwagen, McFadden et al. 1999). Finally, it has been showed that P4 stimulated a decline in TJs proteins Occludin, Cldn-1, -2, -3 and -4, as well as in AJs' proteins E-cadherin and β-catenin in the uterine epithelia suggesting that AJs can also be regulated by hormones (Satterfield, Dunlap et al. 2007). In another study, FSH alone or a combination of FSH and testosterone stimulated the re-organisation of E-cadherin and β-catenin as an extensive continuous belt around periphery of Sertoli cells in culture (Sluka, O'Donnell et al. 2006).

Since hormones occurred to be a master regulator of intercellular junctions in many hormone-dependant organs, perturbation and dysregulation of these key regulators can lead to substantial changes and to the pathologies associated to these shortcomings. Upon industrialisation, nowadays human and many other species are in danger of chronic exposure to wide variety of exogenous substances in every day basis life.

1.6 Endocrine disruptors and Brominated Flame Retardants (BFRs)

The U.S. Environmental Protection Agency (U.S. EPA) Risk Assessment Forum using a number of ecologists, epidemiologists, endocrinologists and toxicologists introduced potential hazardous effects rising from environmental chemicals.

Endocrine Disruptors (EDs) are the widespread range of exogenous substances which perturb the natural hormones functions in homeostasis and normal development. EDs can interfere with the production, release, transportation, binding, action, metabolism or elimination of natural hormones (Mallozzi, Bordi et al. 2016) (Rice, Birnbaum et al. 2003) (Brucker-Davis 1998, Howdeshell 2002). There are some major concerns about EDs;

1) They can be passed to the next generation either via placenta during embryogenesis or during breast feeding of newborns (Polder et al., 2008; Thomsen et al., 2010) (Leonetti, Butt et al. 2016).

2) Compared to classical toxicology studies, EDs effects have been observed at low doses which resemble the human exposure doses (reviewed in (Vandenberg, Colborn et al. 2012).

3) Exposure to EDs typically results in a non-monotonic dose response curves (NMDRCs), meaning that the higher dose does not necessarily lead to more important effects (reviewed in (Vandenberg, Colborn et al. 2012) (Mallozzi, Bordi et al. 2016),

4) Prenatal (in utero) exposures to EDs have been associated to greater incidence of breast cancer (Fenton, Reed et al. 2012) (Jenkins, Betancourt et al. 2012) (Waring, Harris et al. 2016).

There are wide varieties of EDs in our environment. Here are the most common EDs which have been reported to cause the physiological perturbations in human and other organisms;

Dichlorodiphenyltrichloroethane (DDT) which has been used as a pesticide had been biomagnified through the food chain. It was found in high concentrations in most of the species and reports have shown their adverse effect on female reproductive organs (Tiemann 2008).

Phthalates, particularly bis (2-ethylhexyl) phthalate (DEHP) have been used in children toys, cosmetics and medical equipment such as blood bag and catheters. DEHP has been associated to disrupt male reproductive organs (Fisher 2004).

Bisphenol A (BPA) is chemically reproduced in the production of polycarbonate plastics and resins such as plastic bottles and containers and metal cans. BPA has been linked to breast and prostate cancer and reproductive problems such as obesity, early puberty, reproductive problems and cancer (Okada, Tokunaga et al. 2008). Bisphenol S (BPS) and bisphenol F (BPF) have also demonstrated the hormonal activity of BPA (Rochester and Bolden 2015).

Polychlorinated biphenyls (PCBs) which are a class of chlorinated compounds are used in coolants and lubricants. These groups of chemicals immediately has shown their adverse effect on manufactures health as their direct exposure to PCBs increased the rate of skin, brain and liver cancer. Moreover, disturbance of thyroid, obesity of children and increase in risk of diabetes have been also linked to PCBs exposure (Loomis, Browning et al. 1997, Mullerova, Kopecky et al. 2008).

Polybrominated diphenyl ethers (PBDEs) or Brominated flame retardants (BFRs) are a diverse group of chemicals incorporated into many household and commercial goods such as textiles, foams, electronic and vehicles in order to meet fire safety standards reduce the fire hazard and inflammability of the combustible materials (G. Camino 1991, Watanabe and Sakai 2003). The most hazardous issue rising from BFRs is that they do not form covalent bonds with the polymer matrices they are incorporated to, thereby easily leach out to the environment and contribute to human chronic exposure (Besis and Samara 2012). BFRs main routes of exposure are through inhalation and ingestion of dust (Johnson, Stapleton et al. 2010) (Stapleton, Dodder et al. 2005) as well as dietary consumption (Wu, Herrmann et al. 2007) (Fraser, Webster et al. 2009). BFRs are bioaccumulative and detected in

various human tissues and matrices, such as breast milk, adipose tissue, serum and hair (Hites 2004, Zota, Park et al. 2011) (Zota, Linderholm et al. 2013) (Sjodin, Jones et al. 2004) (Toms, Hearn et al. 2009) (Meeker, Johnson et al. 2009).

Among these diverse group of chemicals, the most widespread congeners in North America and U.S. are Polybrominated diphenyl ethers (PBDEs) and Hexabromocyclododecane (HBCD) (Alaee, Arias et al. 2003) (Stapleton, Allen et al. 2008) (Segev, Kushmaro et al. 2009). In 2008 the Canadian Government has prohibited the manufacture, and limited the importation and use of tetraBDE, pentaBDE, hexaBDE, heptaBDE, octaBDE, nonaBDE and decaBDE congeners (Environment 2008). The Canadian governments has adopted similar a legislation for HBCD usage and commerce that should be effective in 2017 (Environment 2010). HBCD and PBDEs are persistent in nature and show bio-magnification through food chain. As a result, human exposure will likely persist for upcoming decades. Consistently, since the above mentioned legislations concerning PBDEs usages, evaluation of their levels in North American domestic house dust revealed that PBDEs levels have not noticeably diminished (Dodson, Perovich et al. 2012).

Another issue rising from BFRs is that certain congeners have been reported to act as an endocrine disruptor (Kim, Harden et al. 2014) by influencing steroid hormones (Turyk et al., 2008), estrogen signaling *in vivo* (Ceccatelli et al., 2006) and *in vitro* (Meerts et al., 2001), androgen signaling *in vivo* and *in vitro* (Stoker et al., 2005) and thyroid hormone signalling and level (Ernest et al., 2012) (Tung et al., 2016). PBDEs mixture (BDE 57, 99 and 100) levels measured in house dust of recruited men was negatively correlated with free androgen index as well as LH and FSH hormones while positively associated with T4 level (Meeker, Johnson et al. 2009). Moreover, high level of certain BFRs congeners (BDEs 28, 47, 99, 100, and 153) was associated with decreased TSH level (Chevrier, Harley et al. 2010).

As mammary glands proper function and development are tightly controlled by hormones, subtle changes can have huge impact and thus render this organ susceptible to EDs (Paulose, Speroni et al. 2015). There are few evidences on the effect of BFRs on mammary gland function and development. Upon a perinatal exposure to DE-71, a significant delay in ductal outgrowth in mammary gland was observed at PND21 (Kodavanti, Coburn et al. 2010). Moreover, an exposure to a PBDEs mixture (47, 99, 100 and 209) combined with estradiol resulted in an increase in proliferation and downturn in apoptosis in MCF7 breast cancer cells (Kwiecinska, Wrobel et al. 2011). However, little is

known about the effect of BFRs on mammary glands function and breast cancer incidence during vulnerable period of development, such as puberty, pregnancy and lactation.

1.6.1 Endocrine disruptors effect on junctional proteins

Since the expression of intercellular junctions can be regulated by hormones, it is postulated that EDs can perturb junctions. There are numerous evidences showing that EDs, pharmaceutical wastes and pesticides abrogated junctional proteins. Hexachlorobenzene exposure decreased Cx43 protein level in MCF-12A breast cells (Delisle, Ferraris et al. 2015) as well as Cx26 and Cx32 in female rat liver (Plante et al. 2002). Upon ioxynil exposure, loss of Cx43 GJs at the plasma membrane, Cx43 mislocalization in intercellular compartments and increased Cx43 degradation were observed in non-tumorigenic rat liver epithelial cells (Leithe, Kjenseth et al. 2010). Similarly, exposure to three pharmaceutical steroids, 17a-ethynylestradiol, medroxyprogesterone acetate and levonorgestrel, led to dysfunctional Cx43 GJs assembly at the membranes of Sertoli cell and to their internalization, but did not affect Cx43 expression levels (Tramoni, Gilleron et al. 2009). Thus, EDs can dysregulate GJs by decreasing connexins expression and/or disruption of GJs channels.

AJs and TJs can also be affected by exposure to EDs, alone or concomitantly to GJs. Bisphenol A (BPA) injection caused simultaneous down-regulation of Cx43 and up-regulation of N-cadherin and ZO-1 in adult male testis (Salian, Doshi et al. 2009). BPA also altered the expression of E-Cadherin and reduced the invasion capability of BeWo cells (Wang, Lu et al. 2015), and induced EMT by increasing N-Cadherin and reduction of E-Cadherin in colon cancer cells (Chen, Yang et al. 2015). Exposure to two widespread EDs steroid compounds, nonylphenol and di-n-butyl phthalate DBP, diminished the expression of TJs proteins occludin, claudin-11 and ZO-1 in primary culture of Sertoli cells (Hu, Wang et al. 2014). Similarly, in human Sertoli cells, a reduction in Occludin, ZO-1 and N-cadherin has been observed after monobutyl phthalate exposure, while BPA reduced the level of Occudin, ZO-1 and β-catenin (de Freitas, Ribeiro et al. 2016). Perfluorooctanoic acid (PFOA) exposure of human choriocarcinoma and endometrial adenocarcinoma in spheroid-endometrial co-culture resulted in suppression of E-cadherin and β-catenin (Tsang, Cheung et al. 2013). Together, these studies suggested that junctional proteins can be targeted by EDs, likely resulting in adverse effects.

1.7 Hypothesis and objectives

From this literature review, important points can be made:

- Mammary gland is a dynamic organ that undergoes substantial changes across the mammals' life span.
- Intercellular junctions are required for providing requisite cell-cell interactions between epithelial cells of mammary gland, and components of Gap, adherens and tight junctions interplay to form a junctional nexus between adjacent cells.
- Alteration, abrogation and mis-localization of junctional proteins can lead to developmental defect and is the hallmarks of breast cancer.
- The mammary gland development is tightly orchestrated by hormones and growth factors.
- Junctional proteins' expression can also be regulated by hormones.
- Endocrine disruptors are defined as compounds that interact with endogenous hormones functions.
- Brominated flames retardants (BFRs) are widespread chemicals. They are bioaccumulative and have been reported to act as endocrine disruptors.

Based on these facts, we hypothesized that the composition of junctional nexus would be remodeled during mammary gland development, and that exposure to endocrine disruptors can dysregulate the expression of junctional proteins, leading to developmental defects.

The two main objectives, and specific sub-objectives, of this research project were thus to:

1) Characterise the dynamic of the junctional nexus across the different stages of mammary gland development by:

1.1) Assessing gap, tight and adherens junctions' protein expression at each specific stage of development;

1.2) Determining the localization of junctional proteins in the junctional nexus in a stage-dependent manner;

1.3) Confirming physical interaction between identified potential binding partners.

Two publications resulted from this objective (see chapter 2):

i. 1st publication; Dianati E, Poiraud J, Weber-Ouellette A and Plante I, *Connexins, E-cadherin, Claudin-7 and β-catenin transiently form junctional nexuses during the post-natal mammary gland development*, Developmental Biology, Aug 2016. PMID: 27291930

ii. 2nd publication; Dianati and Plante, *Analysis of protein-protein interactions and co-localisation between components of gap, tight and adherens junctions in murine mammary glands*, Journal of Visualized Experiments, May 2017. PMID: 28605375.

2) Determine the impact of an exposure to a mixture of Brominated flame retardants (BFRs) on mammary gland development and function, by:

2.1 Analysing mammary gland function, as defined by milk synthesis and associated signaling;

2.2 Evaluating the expression and localisation of markers of the Epithelial-mesenchymal transition (EMT) and of intercellular junctions;

2.3 Assessing β-catenin dual function in cell transduction and adhesion.

One publication resulted from this objective (see chapter 2), and another one, done in collaboration, is in preparation for submission (see annexe):

i. 1st publication; Dianati E, Wade M.G, Hales B, Robaire B and Plante I, Exposure to an Environmentally Relevant Mixture of Brominated Flame Retardants Decreased p-β-catenin^{ser675} Expression and its Interaction with E-cadherin in the Mammary Glands of Lactating Rats *,* June 2017. Accepted for publication in Toxicological Sciences.

ii. 2nd publication; Lavoie M, Dianati E, Wade M, Hales B, Robaire B and Plante I, *A Perinatal Exposure to Brominated Flame Retardants Suppresses E-cadherin and Hormone Receptor α Expression in Mammary Glands at Puberty* (unofficial title, manuscript in preparation)

2 Publications

2.1 Connexins, E-cadherin, claudin-7 and β-catenin transiently form junctional nexuses during the post-natal mammary gland development

Titre en français; Les connexines, cadhérines et claudines forment un complexe jonctionnel transitoire au cours du développement des glandes mammaires chez la souris

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Authors' contribution;

Elham Dianati has done the majority of the experiments, supervised J. Poiraud and A. Weber-Ouellette, participated in experiments design, as well as written and revised the manuscript in collaboration with the corresponding author.

Jérémy Poiraud contributed to immunofluorescence and immunoprecipitation experiments.

Anne Weber-Ouellette contributed to western blot troubleshooting and performed all experiments related to Figure 7A.

Isabelle Plante has supervised the project, contributed to experimental design as well as preparation and revision of this paper.

Abstract

Gap junctions are intercellular channels made of connexins (Cxs) that allow direct communication between adjacent cells. Modulation of Cxs has been associated with abnormal development and function of the mammary gland and breast cancer. However, the mechanisms underlying their expression during normal mammary gland are not yet known. Cxs interact with components of tight and adherens junctions. Thus, we hypothesized that the expression levels of Cxs vary during mammary gland development and are regulated through stage-dependent interactions with members of the tight and adherens junctions. Our specific objectives were to: 1) determine the expression of Cxs and tight and adherens junction proteins throughout development and 2) characterize Cxs interactions with components of tight and adherens junctions. Murine mammary glands were sampled at various developmental stages (pre-pubescent to post-weaning). RT-qPCR and western-blot analyses demonstrated differential expression patterns for all gap (Cx43, Cx32, Cx26, Cx30), tight (claudin-1, -3, -4, -7) and adherens (β-catenin, E- and P-cadherins) junctions throughout development. Interestingly, co-immunoprecipitation demonstrated interactions between these different types of junctions. Cx30 interacted with Cx26 just at the late pregnancy stage. While Cx43 showed a persistent interaction with β-catenin from virginity to post-weaning, its interactions with E-cadherin and claudin-7 were transient. Cx32 interacted with Cx26, E-cadherin and β-catenin during lactation. Immunofluorescence results confirmed the existence of a junctional nexus that remodeled during mammary gland development. Together, our results confirm that the expression levels of Cxs vary concomitantly and that Cxs form junctional nexuses with tight and adherens junctions, suggesting the existence of common regulatory pathways.

1. Introduction

The mammary gland is a unique organ because most of its development occurs after birth. It is composed of two compartments: the stroma, which is also called the fat pad, and the parenchyma or epithelium. The stroma is mainly composed of adipocytes but also contains fibroblasts, endothelial cells, and hematopoietic cells. The epithelium is a tree-like structure composed of ducts and milk-secreting structures termed alveoli. At birth, only a rudimentary epithelial ductal tree is present (Richert et al., 2000). Under the surge of hormones at the onset of puberty (around week 6 in mice), these ducts will begin elongating through highly proliferative structures found at the end of ducts, the

terminal end buds (TEBs), and ramify (Daniel CW, 1987; Atwood et al., 2000; Neville et al., 2002). In adults (around week 10 in mice), most of the fat pad is invaded by this tree-like shape epithelium. A second phase of proliferation and differentiation occurs during pregnancy when the ducts branch in a dichotomous manner to form the alveoli, the milk-secreting units, which remain until the end of lactation. Post-weaning, most of the parenchyma will degrade through epithelial regression and cell apoptosis, which is called involution; consequently, the gland's structure reverts to the non-lactating adult stage (Richert et al., 2000; Sternlicht et al., 2006; Oakes et al., 2014).

The mammary gland epithelium is a bi-layered structure composed of an inner layer of luminal epithelial cells facing a central cavity and an outer layer of basal cells, mainly myoepithelial cells, lying on a basement membrane (Visvader, 2009; Oakes et al., 2014). Normal mammary gland development and function require constant interactions between the stroma and epithelium and between the luminal and myoepithelial cells of the epithelium (Steinet al., 2006; Oakes et al., 2006). These interactions can occur via junctions. Gap junctions (GJs) are a specialized class of channels that are composed of connexins (Cxs), which connect the cytoplasm of two adjacent cells and allow the exchange of ions and molecules less than 1kDa (Maeda, 2009; Maeda and Tsukihara, 2011). Several studies have shown that loss of Cxs is associated with abnormal development and dysfunction of different organs and with different type of cancers (Plante and Laird, 2008; Plante et al., 2010; Gabriel et al., 1998; Sulkowski et al., 1999; Oguro et al., 2001; Teubner et al., 2003). Four different Cxs have been identified in rodent mammary epithelium: Cx26, Cx30 and Cx32 are expressed in luminal epithelial cells, and Cx43 is mainly expressed in myoepithelial cells and at the junction of myoepithelial and luminal cells (Talhouk et al., 2005; Locke et al., 2004; El-Sabban et al., 2003). Importantly, Cxs expression levels vary during mammary gland development, suggesting stage-dependent roles and mechanisms of regulation for each Cx (Locke et al., 2007). Understanding these roles and mechanisms is crucial to understand pathologies linked to dysregulated Cxs.

Studies throughout past two decades have shown that Cxs are not only responsible for the passage of soluble molecules but are also actively involved in cell signaling, mainly by interacting with other proteins through their cytoplasmic tails. Cxs interact and co-immunoprecipitate with a variety of structural and signaling molecules, such as tubulin (Giepmans et al., 2001), zonula occluden-1 (ZO-1), catenins (Ai et al., 2000; Xu et al., 2001), occludin, N-cadherin (Wei et al., 2005) and Src proteins (Duffy et al., 2002); additionally, they co-localize with adherens and tight junctions (AJs and TJs, respectively) (Angst et al., 1997). Thus, GJs, AJs and TJs may form a junctional nexus in which the different

components are regulated by common pathways and might then regulate each other's functions and localization (Ai et al., 2000; Giepmans, 2004; Hertig et al., 1996; Jongen et al., 1991; Kurley et al., 2012; Xu et al., 2001; Ale-Agha et al., 2009; Kanczuga-Koda et al., 2014; Fujimoto et al., 1997).

The major function of AJs is to maintain a physical association between cells, but these junctions are also involved in cell polarity, growth, structure, migration and differentiation (Gumbiner, 1996; arue et al., 1996; Gumbiner, 2005). Thus, their loosening is associated with loss of cell-cell contact, leading to disorganized tissue structure (Schneider and Kolligs, 2015; Breier et al., 2014; van Roy, 2014). In epithelial cells, AJs are mainly formed by two families of proteins: the transmembrane proteins cadherins and the cytoplasmic proteins catenins (Gumbiner, 2005). In the mammary gland, E-cadherin and P-cadherin are expressed in the luminal and myoepithelial cells, respectively (Daniel et al., 1995; Knudsen and Wheelock, 2005). TJs form a semi-permeable barrier between the apical and basolateral domains that prevents the diffusion of lipids and proteins (Tsukita et al., 2001; Itoh and Bissell, 2003). They are necessary for differential permeability in lactating mammary gland, tissue adhesion, migration, polarity, differentiation and homeostasis (Itoh and Bissell, 2003; Lelievre, 2010; Tsukita et al., 2001). They are mainly composed of transmembrane occludin and claudins (Cldns). In the mammary gland, occludin and Cldn-1, -3, -4 and -7 were identified at some or all stages of development (Blackman et al., 2005) and their expression levels may be influenced by lactogenic hormones such as prolactin, glucocorticoids and progesterone (Nguyen et al., 2001; Rubenstein et al., 2003; Stelwagen et al., 1999).

Dysregulation of AJ and TJ proteins has also been associated with developmental defects and cancer in the mammary gland (Hoevel et al., 2004; Osanai et al., 2006, 2007; Lanigan et al., 2009). For example, the β-catenin survival signal is necessary for normal lobular mammary gland development (Tepera et al., 2003), and its inhibition or mis-localization results in hyperplasia and breast cancer (Michaelson and Leder, 2001; Incassati et al., 2010; Lopez-Knowles et al., 2010). E-cadherin and P-cadherin are also essential for the growth of epithelial cells (Lanigan et al., 2007), and their dysregulation is linked to lactation defects (Boussadia et al., 2002); premature differentiation of the gland, resulting in hyperplasia and dysplasia with age (Radice et al., 2003); and breast cancer (Paredes et al., 2005). Finally, a recently identified breast cancer subtype, the claudin-low subtype, is characterized by low levels of proteins that form AJs and TJs and high expression of epithelial-to-mesenchymal transition-associated genes (Prat et al., 2010; Herschkowitz et al., 2007).

In summary, Cxs dysregulation can lead to abnormal mammary gland development and breast cancer. Moreover, the regulation of Cxs in epithelial cells is linked to AJs and TJs in different tissues. Therefore, we hypothesized that a junctional nexus formed by GJs, AJs and TJs is present in the mammary gland and will be remodeled throughout the development to provide the required stage-dependent cell-cell interactions. The objectives of this study were to: 1) determine the endogenous expression pattern of junctional proteins and 2) characterize Cx interactions with components of AJs and TJs during mammary gland development.

2. Material and methods

2.1. Animals

Male and female C57BL/6 mice (8 weeks old) were purchased from Charles River Canada (St. Constant, Quebec, Canada). Mice were maintained under a constant photoperiod of 12 h light: 12 h dark and received food and water ad libitum. All animal protocols used in this study were approved by the University Animal Care Committee (INRS-Institut Armand-Frappier, Laval, Canada). Female mice were monitored each day during two regular estrous cycles by checking vaginal smears and mated during the third estrous cycle (around week 10 or older). Vaginal smears were observed the day after mating for the presence of sperm, and the first day post-mating was considered as the 1st day of pregnancy. Female mice were sacrificed using CO2 followed by cardiac puncture, and the mammary glands were collected at the following time points: pre-pubescent (week 4 (W4)), puberty (W6), adult (W10), pregnancy day 8 (P8), pregnancy day 18 (P18), lactation day 7 (L7), lactation day 14 (L14), involution day 1 (In1) and involution day 8 (In8). For each developmental stage, at least 6 mice were sampled ($N \geq 6$). The mammary glands were preserved differently depending on the downstream applications. Mammary gland pairs 4 and 5 (abdominal and inguinal) were flash-frozen in liquid nitrogen immediately after dissection and used for western blot, RT-qPCR and immunoprecipitation. All samples were stored at -80 °C. Mammary gland pairs 2–3 (upper-thoracic and thoracic) were embedded in VMR frozen sections compound (VWR Inter-national, Ville Mont-Royal, QC, Canada) on dry ice or fixed in the 4% formalin and used for histology and immunofluorescence.

2.2. Masson's trichrome staining

Formalin-fixed tissues were embedded in paraffin, cut at 7 mm and stained using Masson's trichrome. Briefly, tissue sections were de-paraffinized, rehydrated through an alcohol series and then

refixed in Bouin's overnight. Sections were stained sequentially with Weigert's iron hematoxylin (10 min), Biebrich scarlet-acid fuchsin (15 min), phosphomolybdic-phosphotungstic acid (20 min) and aniline blue (5 min) and washed with water between all coloration steps. Finally, the sections were treated with 1% acetic acid for 5 min, dehydrated in an alcohol series, cleared in xylene for 5 min and mounted using Permount (Fisher Scientific, Burlington, ON).

2.3 Western blot

Frozen tissues were ground in liquid nitrogen. The resulting powder was resuspended on dry ice in ice-cold triple detergent lysis buffer (pH: 8) (Tris 50 mM, NaCl 150 mM, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate) that was supplemented with NaF 1.25 M, NaVO3 1 M and Halt Protease and Phosphatase Cocktail Inhibitor (Fisher Scientific). The homogenates were then incubated for 30 min with slow agitation at 4 °C, followed by sonication. Homogenates were centrifuged at Z13000 g for 10 min at 4 °C, and the supernatants were aliquoted and stored at - 80 °C until later usage. Protein content was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, Illinois, USA). To perform semi-quantitative western blot analysis, between 10 and 50 mg of total protein (Taylor et al., 2013) was loaded onto TGX Stain-FreeTM Acrylamide gels (BIO-RAD, Mississauga, Ontario, Canada). Immediately after electrophoresis, the gels were transferred onto PVDF membranes (Trans-blot turbo RTA Transfer kit, BIO-RAD) using the Trans-Blot Turbo Transfer System (BIO-RAD). Total lane proteins were visualized using the ChemiDoc MP imaging system (BIO-RAD) and quantified using ImageLab 5.2 software (BIO-RAD) for normalization. Membranes were blocked with 5% dry milk or 3% BSA in TBS-Tween 0.1% and probed overnight with one of the following primary antibodies: β-catenin (D10A8) rabbit mAb 1/1000 (#8480s), claudin-1 (#4933s) 1/1000, E-cadherin (24E10) rabbit mAb 1/1000 (#3195s), phos-phPlus Stat3 (Tyr 705) (#9130), phospho-Stat5 (Tyr 694) rabbit mAb (#4322s), or Stat5 rabbit Ab (#9363s) from Cell Signaling (Beverly, MA, USA); claudin-3 (#34–1700) 1/1000, claudin-4 (#36–4800) 1/1000, claudin-7 (#34–9100) 1/1000, connexin-26 (#33–5800) 1/500, Connexin-30 (#71–2200) 1/500 from Life Technologies/Invitrogen (Waltham, MA, USA); Connexin32 (#265-279) 1/1000 or Connexin43 (#C6219) 1/1000 from Sigma-Aldrich (Oakville, Ontario); and P-cadherin 6A9 (#MA1-2003) 1/5000 from Thermo Scientific (Mississauga, ON, Canada). Membranes were washed with TBS-Tween 0.1% and probed with 2nd anti-bodies (anti-rabbit IgG HRP-linked antibody (#7074s) or anti-mouse IgG HRP-linked antibody (#7076s) (Cell Signaling). The signal was obtained using ClarityTM Western ECL Blotting Substrate (BIO-RAD) and visualized using the ChemiDoc MP imaging system (BIO-RAD). The density of each band was normalized to the total lane proteins using ImageLab 5.2 software.

2.4 Immunofluorescence analysis and microscopic image acquisition

Tissue cryosections (7 mm) were fixed in formaldehyde 4% and blocked in 3% BSA dissolved in TBS-Tween 0.1%. Primary antibodies were diluted in either blocking or TBS-Tween 0.1% solutions, and sections were incubated with antibody for 60 min at room temperature or overnight at 4 °C. Sections were incubated with E-cadherin mAb Alexa Fluors555 Conjugated (#4295s) 1/50 (Cell Signaling), β-catenin (L54E2) mouse mAb (#2677s) 1/200 (Cell Signaling), Connexin26 (#33-5800) 1/75 (Life Technologies), claudin-3 1/100 (#341700) (Life Technologies), Cytokeratin 14 Ab-1 mouse monoclonal antibody 1/800 (#LL002) (Thermo Scientific), Connexin43 (#C6219) 1/500 (Sigma-Aldrich), Connexin32 (#265-279) 1/100 (Sigma-Aldrich), P-cadherin (6A9) MA-2003 1/500 (Thermo Scientific), Connexin30 (#71-2200) 1/75 (Life Technologies), claudin-4 (#36-4800) 1/50 (Life Technologies), or claudin-7 (#34-9100) 1/100 (Life Technologies). Staining was followed by three washes with TBS-tween 0.1%. Sections were incubated with the appropriate secondary antibodies (anti-rabbit IgG Fab2 Alexa Fluor 488 (#4412s), anti-mouse IgG Fab2 Alexa Fluor 647 (#4410 s), or anti-mouse IgG Fab2 Alexa Fluor 555 (#4409s), all used at 1/1000 from Cell Signaling or donkey anti-rabbit IgG antibody, Alexa Fluors 568 conjugate (A10042), used at 1/1000 from Life Technologies. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI), and slides were mounted with Fluoromount-G (Cedarlane, Burlington, ON). Immunofluorescence images were obtained with a Nikon A1Rþ confocal microscopic laser equipped with a spectral detector and analyzed using NIS-elements software (version 4).

2.5. RNA extraction, primer validation and RT-qPCR

Total RNA was extracted from flash-frozen tissues using AurumTM Total RNA Fatty and Fibrous Tissue Kit (BIO-RAD) following the manufacturer's instructions. The RNA concentration was quantified with the Nanodrop1000 (Thermo Scientific), while purity and integrity were assessed using Experion RNA StdSens Analysis Kit using the ExperionTM automated Electrophoresis System (BIO-RAD). RNA was converted to cDNA using an iScriptTM cDNA Synthesis Kit (BIO-RAD). To obtain the ideal cycle threshold (CT), different concentration of cDNA (Lanoix et al., 2012) was amplified using SsoFastTM EvaGreens Supermix (BIO-RAD) following the manufacturer's instructions. Gene expression was quantified using Bio-Rad CFX manager software (edition 3.1). The expression levels of the genes of interest were normalized to reference genes. Reference genes were selected based on their stability using geNorm biogazelle qbase+ software (Zwinaarde, Belgium). The primer sequences are listed in supplementary Table 1.

2.6. Immunoprecipitation

The lysates used for immunoprecipitation were prepared from flash-frozen tissue as described above. Immunoprecipitation was performed using the PureProteomeTM Protein G Magnetic Bead System (LSKMAGG02) (Millipore, Etobicoke, ON, Canada) following the manufacturer's instruction. Briefly, 200 ml of lysates (500 µg-1 mg) and antibodies (rabbit (DA1E) mAb IgG Isotype control 0.5 ml/ 200 µl (#3900 s) (Cell Signaling), mouse (G3A1) mAb IgG Isotype control 0.5 µl / 200 µl (#5415 s) (Cell Signaling), Connexin43 4 µl / 200 µl (#C6219) (Sigma-Aldrich), Connexin32 (#265–279) (Sigma-Aldrich), or Connexin30 5 µl /200 µl (#700–258) (Life Technologies)) were incubated overnight at 4 °C with slow agitation. The next day, 50 ml of magnetic beads was added to the lysate-antibody solution, and the samples were incubated at room temperature for 75– 90 min with slow agitation. Beads were retained with a magnet, and the flow through was discarded. After washes, the bead-antibody-protein complexes were dissociated using two consecutive incubations with 30 µl of 0.2 M glycine (pH=2.5) to remove the IgG. Then, 15 µl of 4x Laemmli Sample Buffer (BIO-RAD) complemented with β-mercaptoethanol was added to the tubes, and the acidic reaction was naturalized by adding 1 M Tris (pH=8). Immunoprecipitation was followed by SDS gel electrophoresis and transfer to PVDF membrane as described above. Blots were probed with the same primary antibodies listed above and then incubated with HRP-conjugated Veriblot for IP secondary antibody 1/5000 (Abcam, Toronto, ON, Canada).

3. Results

3.1. Time-points are representative of stages of post-natal mammary gland development

To verify that the chosen time points correspond to the appropriate stage of development, we first performed Masson's trichrome staining (Fig. 1). As expected, the gland was mainly composed of adipocytes and few epithelial structures at W4 (Fig. 1). From W6 to W10, the epithelial cells proliferated, and more ducts were found within the gland. At P8, alveoli buddings started to appear. At P18, L7 and L14, the stroma was filled with alveoli, and milk was observable in the lumen of few alveoli at L7 and L14 (Fig. 1). One day after weaning (In1), the epithelium began degrading, and the gland fully regressed to the non-lactating adult phase by In8 (Fig. 1). These data confirm that the chosen time points represent the different stages of mammary gland development.

3.2. Gap junction proteins, Cx43, Cx32, Cx26 and Cx30 are differentially expressed throughout mammary gland development in mice

Four Cxs were previously identified in the murine mammary gland: Cx43, Cx32, Cx26 and Cx30 (Monaghan et al., 1994; Talhouk et al., 2005). To shed light on the modulation of gap junction expression in the mouse mammary gland, Cxs mRNA and protein levels were measured by RT-qPCR and western blot analyses, respectively (Figs. 2 and 3). Our results showed that the Cx43 (Gja1) mRNA levels increased from W4 to W6, not changed from W6 to W10 and rose again from W10 to P8. Gja1 levels remained un-changed between P8 and P18, followed by a sharp reduction during lactation, and a significant up-regulation during involution (Fig. 2(A)). In contrast, Cx26 (Gjb2) was not detectable between W4 and P8, but its expression peaked at P18 and was followed by a sharp 3–4 folds drop at L7, L14 and In1 (Fig. 2(B)). It significantly decreased again at In8 (Fig. 2(B)). Cx30 (Gjb6) transcripts were undetectable from W4 to P8. At high elevation was observed at P18, followed by a 4-fold decrease at L7 and L14, and then transcripts went back to barely detectable levels during involution (Fig. 2(C)).

For Cx43, Cx26 and Cx30, the mRNA (Fig. 2(A)-(C)) and protein (Fig. 3(A)-(C)) levels mostly followed the same trends, suggesting that the observed variations were due to change in gene transcription. Cx32 (Gjb1) mRNA levels were barely detectable in virgin animals and during pregnancy, peaked at L7 and then dropped by two folds at L14 (Fig. 2(D)). The transcription decreased to virgin levels at the time of involution (Fig. 2(D)). The protein expression pattern was quite similar to the mRNA level, although low to medium levels of Cx32 could be detected at P8 and P18, when the transcript level was low (Fig. 3(D)). These results suggest that the Cx32 transcript or protein is stabilized at those stages, followed by an up-regulation in gene transcription during lactation.

Together, these results show that Cxs demonstrate a differential expression pattern throughout mammary gland development (Figs. 2 and 3), suggesting the existence and necessity of different regulatory mechanisms for each Cx throughout development.

3.3. Tight and adherens junction proteins show a stage-dependent expression pattern during mammary gland development

To verify whether Cxs can be regulated via protein interactions with components of TJs and AJs, we first needed to determine the expression patterns of those proteins.

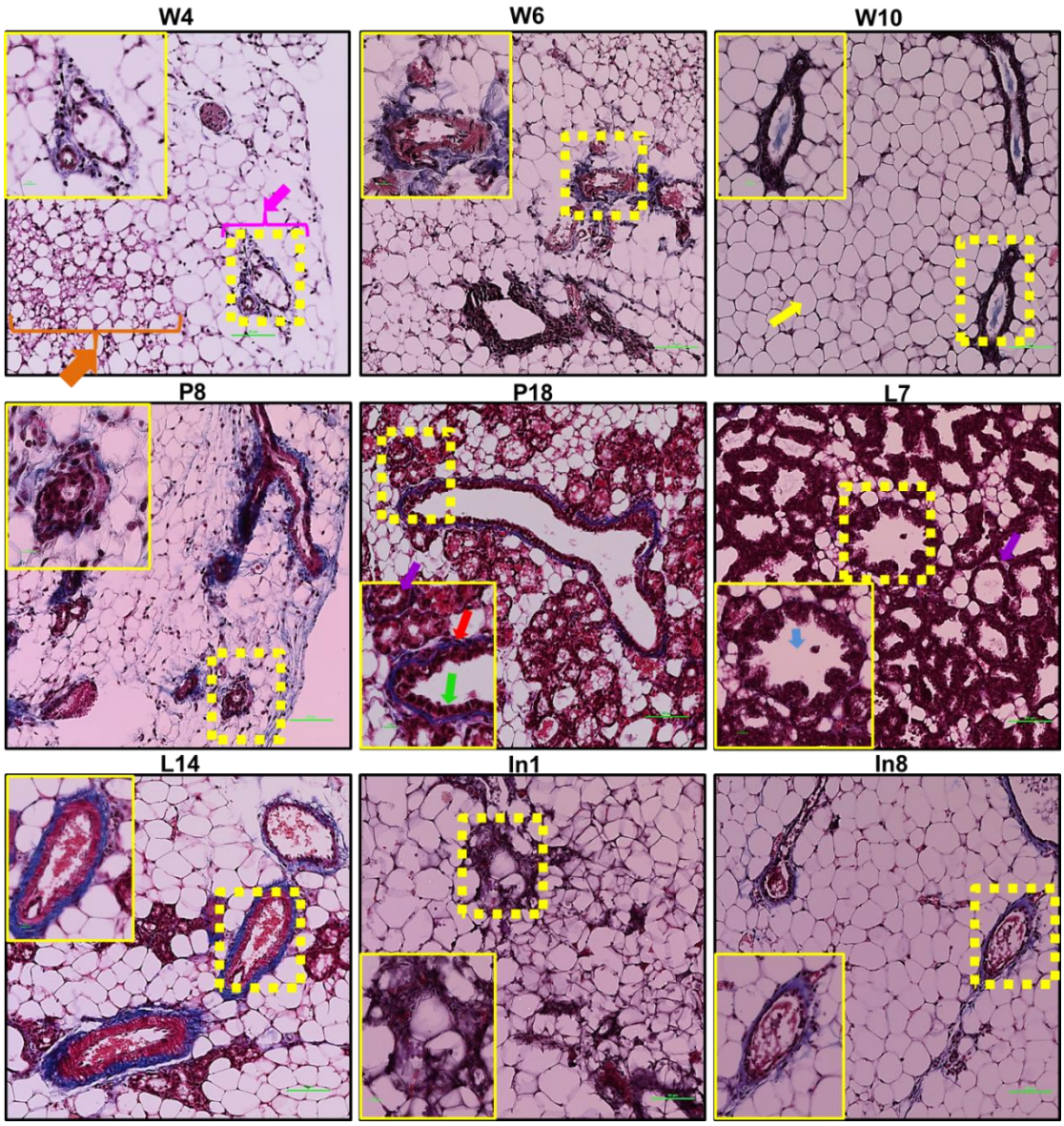

Fig. 1. The mammary gland acquires different morphology throughout post-natal development in mice. Paraffin-embedded murine mammary glands from different developmental stages (before mating (W4, W6, W10), during pregnancy (P8, P18), lactation (L7, L14) and involution (In1, In8)) were sectioned (7 mm) and stained using Masson's trichrome. Representative images are shown. Nuclei are stained in dark purple, the cytoplasm is stained in red, and connective tissues (myoepithelial cells and collagen) are stained in blue. Example of mammary glands structures are identified with colored arrows; Adipocytes (yellow), stroma (orange), myoepithelial cells (red), luminal epithelial cells (green), lumen (blue), alveoli (purple).

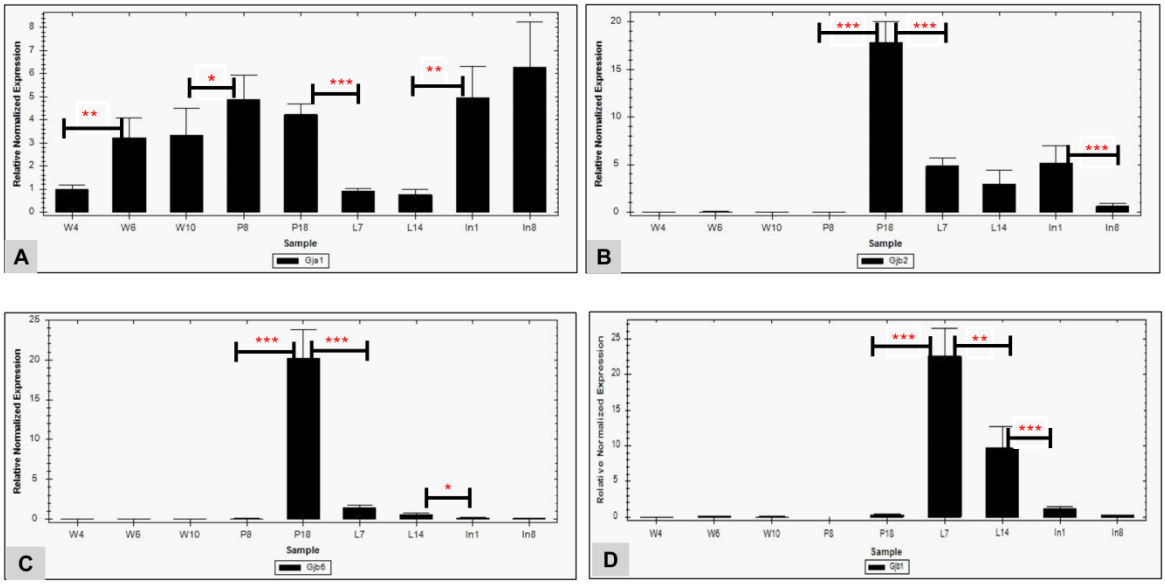

Fig. 2. Cx43, Cx26, Cx30 and Cx32 mRNA levels exhibit stage-dependent changes during mammary gland development. Total mRNA was extracted from murine mammary glands before mating (W4, W6, W10) and during pregnancy (P8, P18), lactation (L7, L14) and involution (In1, In8); the mRNA was then subjected to RT-qPCR. The graphs present the mean±SEM of expression of Cx43 (A), Cx26 (B), Cx30 (C) and Cx32 (D) after normalization to Gapdh and Atp5b (N=4) using Bio-Rad CFX Manager 3.1 software. *P <0.05, **P < 0.01, ***P <0.001.

To evaluate TJ components, we measured the expression of Cldn-1, -3, -4 and -7 at the mRNA and protein level (Figs. 4 and 5). Cldn-1 mRNA levels gradually increased from W6 to P18, sharply decreased at L7, raised slightly at L14, peaked at In1 and then dropped by 2–3 fold at In8 (Fig. 4(A)). Cldn-3 transcript levels increased after puberty and remained stable until P18 when a six-fold raise is observed (Fig. 4 (B)). Similar to Cldn-1, Cldn-3 mRNA levels significantly dropped during lactation, peaked at In1 and significantly decreased at In8 (Fig. 4(B)). Cldn-4 mRNA levels gradually increased from W4 to W10, decreased at P8, significantly increased at P18, decreased again during lactation, peaked at In1 and dropped back to non-lactating adult level at In8 (Fig. 4(C)). Cldn-7 transcript levels increased slightly at puberty, were not different between W6 and W10, significantly raised at P8, followed by another 3–4 fold raise at P18 (Fig. 4(D)). No statistically significant changes were observed between P18 and L7, L14, In1 and In8 (Fig. 4(D)).

Notably, protein levels did not follow the same trends for Cldn-1; the western blot analysis showed similar levels of expression of Cldn-1 throughout all developmental stages (Fig. 5(A)). The Cldn-3, Cldn-4

and Cldn-7 protein levels showed similar trends as their mRNA transcripts across most the developmental stages, except for Cldn-3. Despite the observed down-regulation at lactation and up-regulation at In1 in mRNA levels, the proteins levels were constant (Fig. 5(B) and (D)). The observed protein stabilization for Cldn-3 and Cldn-7 suggests a functional necessity of these proteins during lactation.

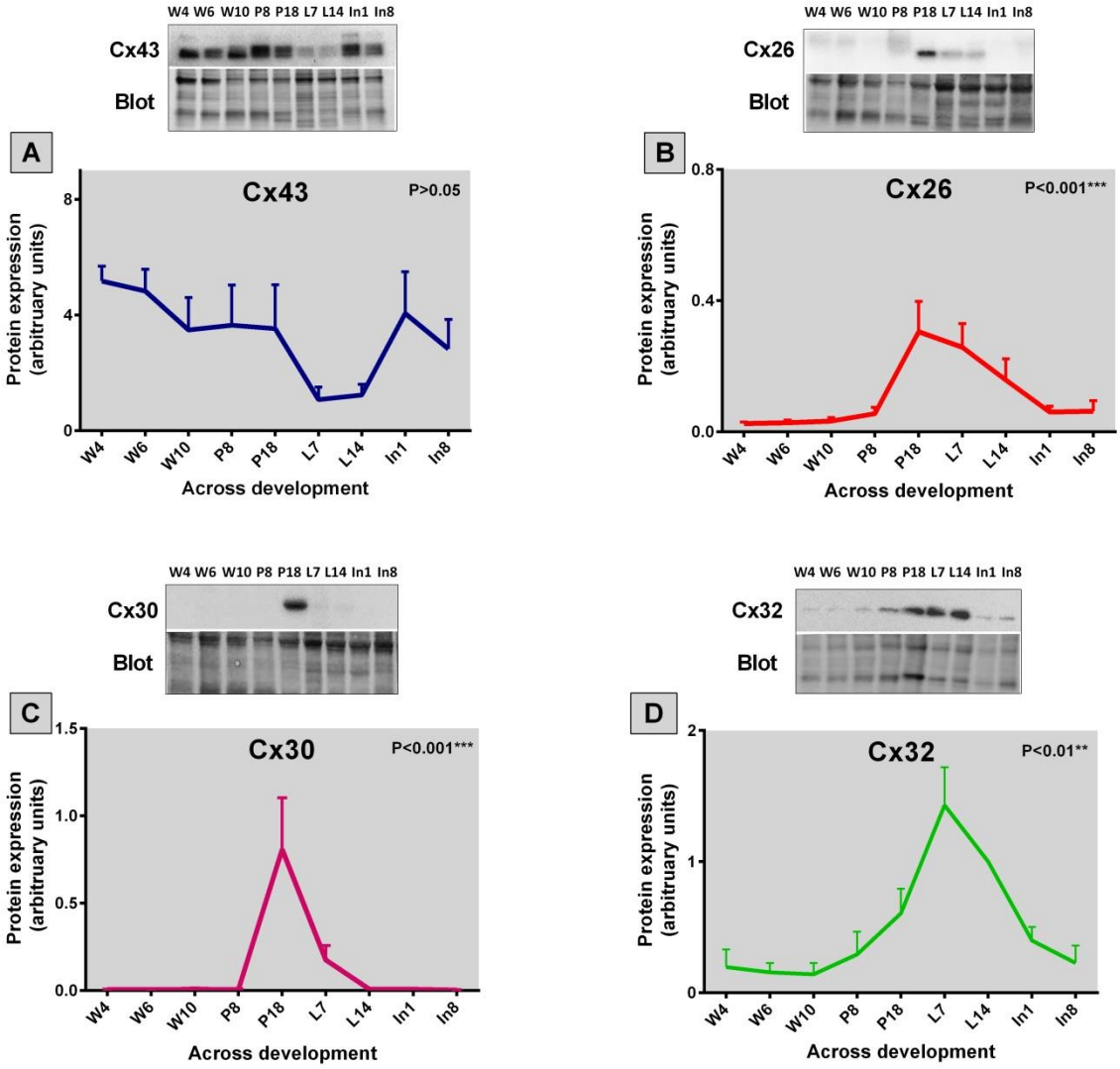

Fig. 3. Cx43, Cx26, Cx30 and Cx32 protein levels exhibit stage-dependent changes during mammary gland development. Total protein was extracted from the mammary glands of virgin (Week 4, Week 6, Week 10), pregnant (Day 8, Day 18), lactating (Day 7, Day 14) and involuting (Day 1, Day 8) mice. Protein expression was measured by western blot for Cx43 (A), Cx26 (B), Cx30 (C) and Cx32 (D). The graphs present the mean ± SEM for each band, normalized to the total protein level (N=4). *P<0.05, **P<0.01, ***P<0.001.

To characterize the expression of AJ components, we analyzed the mRNA and protein levels of E-cadherin and P-cadherin, the two main transmembrane AJ proteins expressed in luminal and myoepithelial cells, respectively, and the key cytoplasmic protein β-catenin at different stages of post-natal development. E-cadherin, P-cadherin and β-catenin showed similar expression pat-terns at the transcript levels: their levels were low or undetectable before puberty, increased during puberty and remained constant in non-lactating adult mice (Fig. 6). At P8, E-and P-cadherin levels gradually increased; however, all three AJ proteins reached a first peak at P18, significantly decreased during lactation, reached a second peak at In1 and remained high at In8 (Fig. 6).

At the protein level, E-cadherin and β-catenin exhibited a similar pattern: levels were low in virgin mice until the beginning of pregnancy (P8), peaked in late pregnancy (P18) and then gradually decreased throughout lactation and involution, although E-cadherin's protein level decreased less rapidly than β-catenin (Fig. 7 (A) and (C)). In contrast, the P-cadherin level increased sharply from W6 to P8, dropped at P18 and then gradually rose from L7 to In8 (Fig. 7(B)). These results suggest that AJs are regulated differently in the luminal and myoepithelial cells. Moreover, our data demonstrate that, similar to Cxs, AJ and TJ proteins also show a stage-dependent regulation pattern.

3.3. Connexins, cadherins and claudins typically located at cell-cell junctions throughout mammary gland development

To evaluate possible interactions between the different junctional proteins, we next evaluated their localization at different stages using immunofluorescence analysis. Cx43, Cx26, Cx30 and Cx32 demonstrated punctate expression patterns at the cell membrane, which is typical for gap junction proteins (Fig. 8). Cx43 was present throughout mammary gland development, mainly between myoepithelial cells, where it co-localized with cytokeratin 14 and P-cadherin (Fig. 8(A), (E) and Supplementary Fig. 1). Cx43 was also expressed between luminal cells and at the junction between luminal and myoepithelial cells (Fig. 8(A) and (E)). Cx26, Cx30 and Cx32 were localized between luminal cells (Fig. 8(B)-(D)). Cx26 was detectable from P18 until In1 (Fig. 8(B)), and Cx30 was detectable from P18 to L7 (Fig. 8(C)). Cx32 was detectable in intracellular compartments at P18 and between luminal cells from L7 until In1 (Fig. 8(D)).

Then, we evaluated the localization of TJ and AJ components (Fig. 9). Cldn-3 and -7 showed similar localization patterns, forming a belt-like structure around luminal cells at W10 and P8 (Fig. 9(A), (C) and

Supplementary Fig. 3). In late pregnancy (P18), the same belt-like structure was observed for both Cldns around the luminal cells of the ducts, but a more apical localization was observed in the acini (Fig. 9(A) and (C)). At L7, Cldn-3 was present at both the apical surface of the luminal cells and in a belt-like structure, while Cldn-7 was no longer detectable at the apical surface.

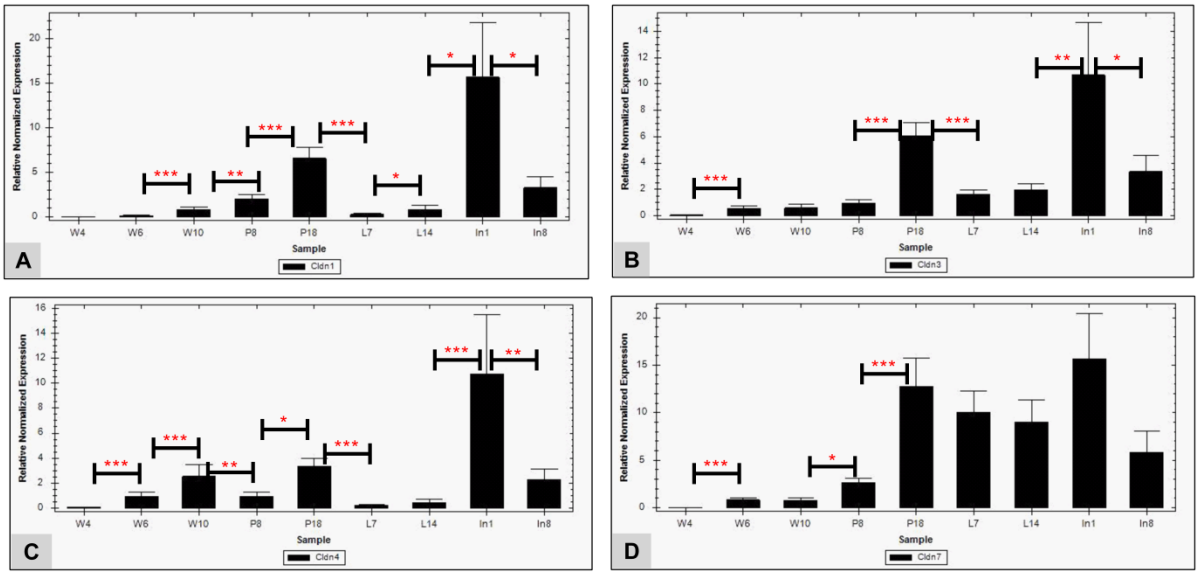

Fig. 4. Tight junction gene expression varies throughout mammary gland development. Total mRNA was extracted from murine mammary gland before mating (W4, W6, W10) and during pregnancy (P8, P18), lactation (L7, L14) and post-weaning (In1, In8); the mRNA was then subjected to RT-qPCR. The graphs present the mean±SEM of expression for Cldn1 (A), Cldn3 (B), Cldn4 (C) and Cldn7 (D) after normalization to Gapdh and Atp5b (N=4) using Bio-Rad CFX Manager 3.1 software. *P<0.05, **P<0.01, ***P<0.001.

At In1, the apical localization of Cldn3 and −7 was disrupted, and the proteins mostly resided at the basolateral side of cells, similar to what was observed in virgin mammary glands (Fig. 9(A) and (C)). Cldn-4 localized around luminal cells at both the apical and basolateral cell surfaces, and the localization was more apical at P18, like Cldn-3 and -7 (Fig. 9(B)). Cldn-4 was almost undetectable at L7 and is localized at both the cell mem-brane and into the cytoplasm upon its re-expression at L14 and In1 (Fig. 9(B)). At In8, the localization was similar to the non-lactating adult mammary glands (Fig. 9(B)).

E-cadherin was also expressed in luminal cells at the plasma membrane from W10 to P18 (Fig. 9(D)). Surprisingly, E-cadherin demonstrated both cytoplasmic and membranous expression patterns

from lactation to In1 (Fig. 9(D) and Supplementary Fig. 2). The localization then reversed to the plasma membrane localization at In8 (Fig. 9(D)). β-catenin localized at both basolateral and apical surface of epithelial cells during all stages of mammary gland development, however it is mostly located at basolateral surfaces at L7 (Fig. 9(E)). Finally, P-cadherin was expressed at the plasma membrane of basal cells and at high levels in the stroma from W10 to P18 (Fig. 9(F) and Supplementary Figs. 1 and 3). From L7 to In1, strong cytoplasmic staining was observed in both the stroma and the epithelium (Fig. 9(F)). Similar to all other Caudins, E-Cadherin and β-catenin, P-cadherin localization at In8 reversed back to the pattern observed at W10 (Fig. 9(F)).

These results show that some of the GJ, TJ and AJ proteins demonstrate similar stage-specific expression patterns, especially during pregnancy and lactation. Thus, interactions between them are possible but require confirmation by other techniques.

3.4. Connexins interact with members of tight and adherens junction protein in a stage-specific manner

We next performed immunoprecipitation analysis to determine whether Cxs interacted with the components of TJs and AJs during mammary gland development. First, Cx43 was immunoprecipitated using mammary gland lysates that were collected at different developmental stages (Fig. 10(A)). We observed that β-catenin persistently interacted with Cx43 at all stages of development, starting before puberty (W4) to the end of involution (In8) (Fig. 10(A)). However, Cx43 demonstrated a transient interaction with E-cadherin that was stronger at P18, L7, L14 and In1 (Fig. 10(A)). The interaction between Cx43 and E-cadherin was confirmed by E-cadherin reciprocal immunoprecipitation (Supplementary Fig. 4). Cx43 also interacted with claudin-7 starting from the non-lactating adult phase to In1, and the strongest interaction was observed during late pregnancy and lactation. This interaction was detectable at In1 but was undetectable by In8 (Fig. 10(A)). No interactions were observed with P-cadherin or with claudin-3 (data not shown).

To determine whether Cx30 resides in a protein complex with other junction proteins, immunoprecipitation analysis was per-formed using lysates from P8, P18 and L7 because Cx30 was not detectable at the other developmental stages by western blot analysis (Fig. 3(C)). Cx30 interacted with Cx26 during P18, when both proteins are highly expressed, and at L7, when Cx30 expression is low (Fig. 10(B)). No interaction was observed between Cx30 and E-cadherin, β-catenin, Cldn-3, Cldn-7 or Cx32 (data not shown).

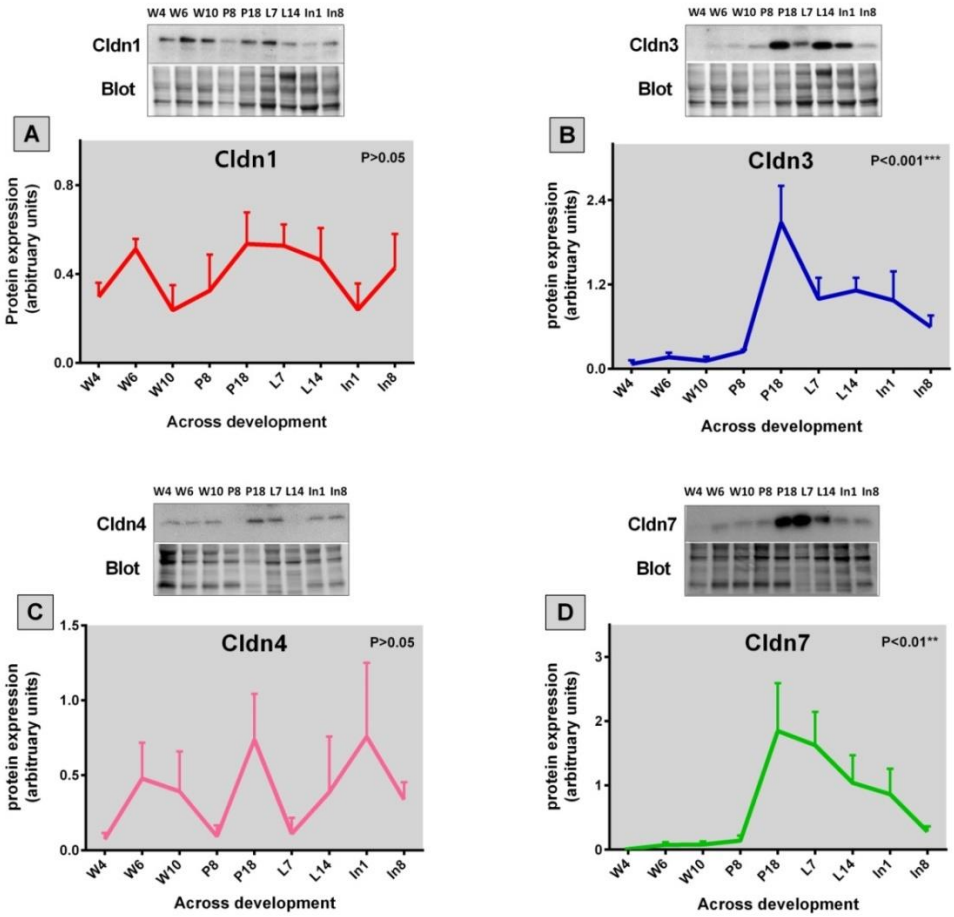

Fig. 5. Tight junction protein levels are modulated during mammary gland development in mice. Total protein extracted from the mammary glands of virgin (Week 4, Week 6, Week 10), pregnant (Day 8, Day 18), lactating (Day 7, Day 14) and involuting (Day 1, Day 8) mice. Protein expression was measured by western blot for Cldn-1 (A), Cldn-3 (B), Cldn-4 (C) and Cldn-7 (D). The graphs present the mean±SEM for each band normalized to the total protein level (N=4). *P<0.05, **P<0.01, ***P<0.001.

Finally, because Cx32 was only localized at the plasma membrane during lactation (Fig. 8(D)), immunoprecipitations were performed using lysates from samples collected at L7 and L14. We observed that Cx32 interacted with Cx26, E-cadherin and β-catenin during both lactation time points (Fig. 10(C)).

Together, our results demonstrate the presence of stage-de-pendent junctional nexuses with different compositions during mammary gland development.

4. *Discussion*

Mammary gland structure constantly changes throughout a mammal's post-natal lifespan. Gap junctional intercellular communication (GJIC) plays a crucial role to promote the proper development and normal function of glands during these morphological changes. In this study, we showed that GJs form distinct complexes with AJ and TJ components throughout mammary gland development, which suggest stage-dependent requirements and mechanisms of regulation.

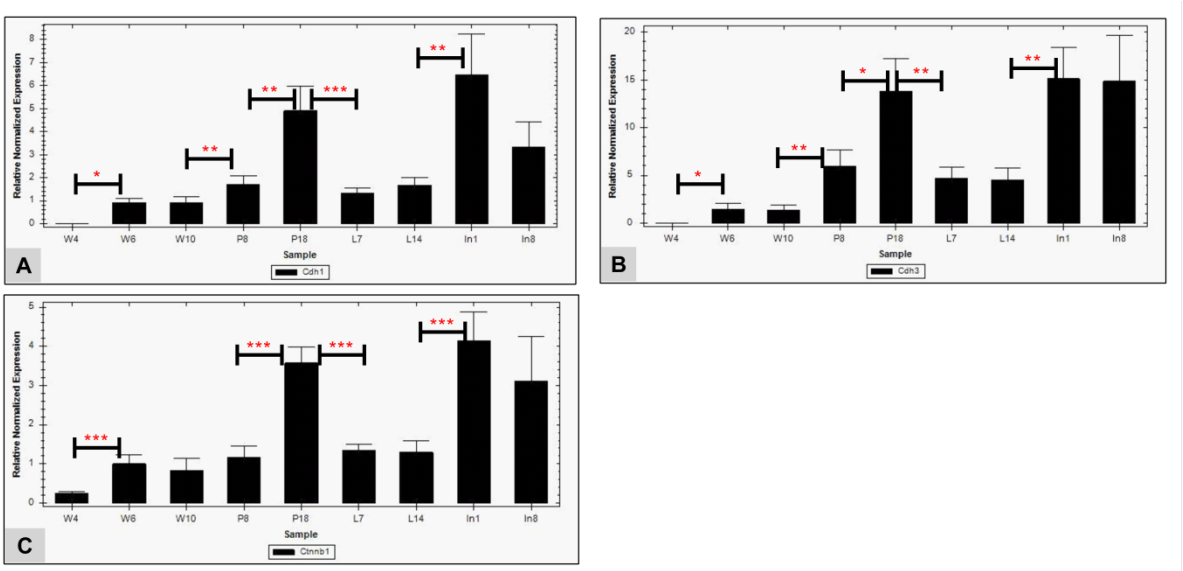

Fig. 6. Adherens junction gene expression varies throughout mammary gland development. Total mRNA was extracted from murine mammary glands before mating (W4, W6, W10) and during pregnancy (P8, P18), lactation (L7, L14) and involution (In1, In8); the mRNA was then subjected to RT-qPCR. The graphs present the mean ± SEM for the expression of Cdh1 (A), Cdh3 (B), Ctnnb1 (C) normalized to Gapdh and Atp5b (N=4) using Bio-Rad CFX Manager 3.1 software. *P<0.05, **P<0.01,***P<0.001.

4.1. *Cx43, Cx26, Cx30 and Cx32 are differentially expressed in the mammary gland throughout development*

Four Connexins, Cx43, Cx32, Cx26 and Cx30, are expressed in murine mammary glands. Few studies have investigated their expression during mammary gland development. Cx43 is always expressed throughout mammary gland development, and its active phosphorylated forms are up-regulated during lactation, while mRNA levels are decreased (Talhouk et al., 2005). Our results confirmed that Cx43 is expressed at all stages at both the gene and protein level, with a similar strong down-regulation in the transcripts at lactation day 7 and 14. However, we did not observe any significant stage-dependent

changes in Cx43 phosphorylation; instead, we observed a strong down-regulation of the total protein level during lactation. The difference might be related to the use of two different strains of mice (C75BL/6 versus BALB/c). C57BL/6 mice exhibit reduced progesterone-induced side-branching and alveologenesis compared with BALB/c mice (Aupperlee et al., 2009). Whether these developmental discrepancies impact or are linked to Cx43 phosphorylation status is unknown.

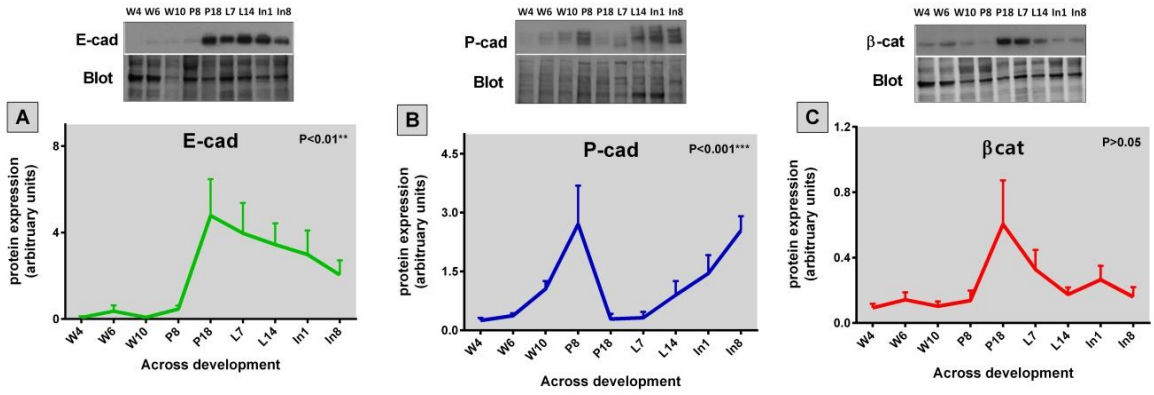

Fig. 7. The E-cadherin, P-cadherin and β-catenin protein levels are modulated during mammary gland development in mice. Total protein was extracted from mammary glands of virgin (Week 4, Week 6, Week 10), pregnant (Day 8, Day 18), lactating (Day 7, Day 14) and involuting (Day 1, Day 8) mice. Protein expression was measured by western blot for E-Cad (A), P-Cad (B), and β-Cat (C). The graphs present the mean±SEM for each band normalized to the total protein level (N=4). *P<0.05, **P<0.01, ***P<0.001.

We confirmed that Cx43 is mainly expressed between myoepithelial cells at all stages. However, similar to what has been shown by others (Talhouk et al., 2005; Mroue et al., 2015), our immunofluorescent studies demonstrated that Cx43 is also present at the junctions between luminal and myoepithelial cells and between luminal cells. These results suggest that Cx43 may interact with junctional proteins that are expressed in both layers of cells and may also contribute to a junctional nexus that links the luminal and myoepithelial cells.

Our results confirmed that Cx26, Cx32 and Cx30 are expressed in luminal cells in a stage-dependent manner (Monaghan et al., 1994; Locke et al., 2004; Talhouk et al., 2005; Locke et al., 2007). We showed that Cx26 is up-regulated at the gene and protein levels during late pregnancy (P18) compared with virgin mice and early pregnancy (P8), followed by a decline at L7 and L14. A similar pattern has been reported at the protein level (Monaghan et al., 1994; Talhouk et al., 2005) and the transcript level using micro-array (Locke et al., 2007). In our study, however, the Cx26 transcript and protein levels remained

low at the beginning of involution (In1) compared with P18, whereas others observed a second expression peak at the transcript level in BALB/c mice using microarray analysis, followed by a sharp decrease at day two of involution (Locke et al., 2007). Whether this delayed decreased compared with our results is linked to the mouse strain or the method of analysis remained to be determined. Nevertheless, these results suggest that Cx26 is mainly required from mid-to late-pregnancy and, to a lesser extent, during lactation.

A previous study reported that Cx30 was expressed at day 15 of pregnancy, peaked at the onset of lactation and then declined (Talhouk et al., 2005). In another report using microarray analysis, Cx30 transcripts were detected from P8.5 in Balb/c mammary glands, peaked at P17.5 and declined during lactation (Locke et al., 2007). In our study, we could not detect Cx30 by RT-qPCR at P8 in C57BL/6 murine mammary glands; however, we observed a comparable expression peak at P18, followed by a decrease at L7. Together, these results suggest that Cx30 expression is required at the end of pregnancy/onset of lactation.

The Cx32 transcript was previously reported to be expressed only during lactation, with a sharp decline after weaning (Locke et al., 2004,, 2007), while another study demonstrated that Cx32 protein expression was detectable during pregnancy, with a sharp increase during lactation (Talhouk et al., 2005).In our study, Cx32 transcripts were barely detectable at the end of pregnancy, peaked at L7 and then slowly decreased to the virgin level at involution. Similar to previous research (Talhouk et al., 2005), we could detect non-negligible levels of Cx32 by western blot during pregnancy, even though the transcripts levels were low, which suggests that the proteins get stabilized during pregnancy. Cx32 was consistently detectable by immunofluorescence mainly in the intracellular compartment during pregnancy and in gap junction plaques during lactation. This result suggests that Cx32 is stabilized at the end of pregnancy in preparation for lactation. Finally, similar to previous studies, Cx26, Cx30 and Cx32 were localized at the interface of two luminal cells, with no evidence of expression in myoepithelial cells. Together, our results confirm that Cx26, Cx30, Cx32 and Cx43 expression is stage-dependent during the mammary gland development. Because the type of connexins that form a channel influence which type of molecules can pass through the channel (Laird, 2006), the connexins' stage-dependent expression suggests different cell-cell communication requirements during mammary gland development. GJs can interact with components of the AJs and TJs in other tissues. Interactions between components of the GJs, AJs and TJs are thought to be important for stabilization and turnover of GJs at the plasma membrane and for cell signaling (Laird, 2010). Our aim was thus to determine

whether Cx26, Cx30, Cx32 and Cx43 could form different junctional nexuses with components of the AJs and TJs, thereby regulating cell-cell interactions.

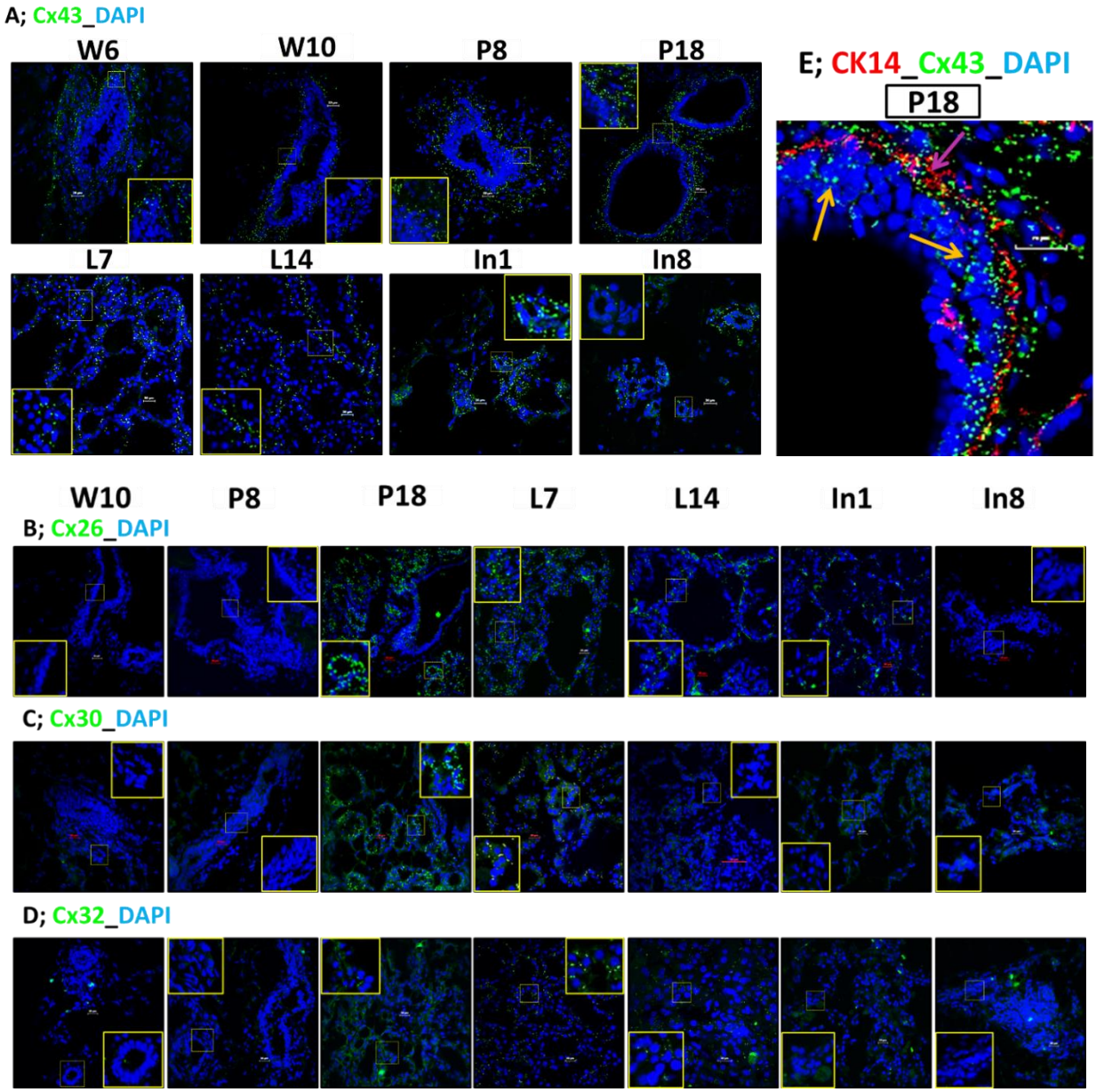

Fig. 8. The localization of Cx43, Cx26, Cx30 and Cx32 during different stages of mammary gland development. Cryosections were cut (7 mm) and processed for immuno-fluorescent staining. A) Cx43 (green) is expressed in the stroma, in myoepithelial cells and between the myoepithelial and luminal cells. Cx43 is also detected at the interface between luminal cells (yellow arrows). E) Cx43 (green) and cytokeratin 14 (red) co-localized in myoepithelial cells (violet arrow). B-D) Cx26, Cx30 and Cx32 (all in green) are expressed at the interface between luminal cells. Nuclei are stained with DAPI. Images were obtained with a Nikon A1Rþ equipped with a spectral detector and analyzed using NIS-elements software.

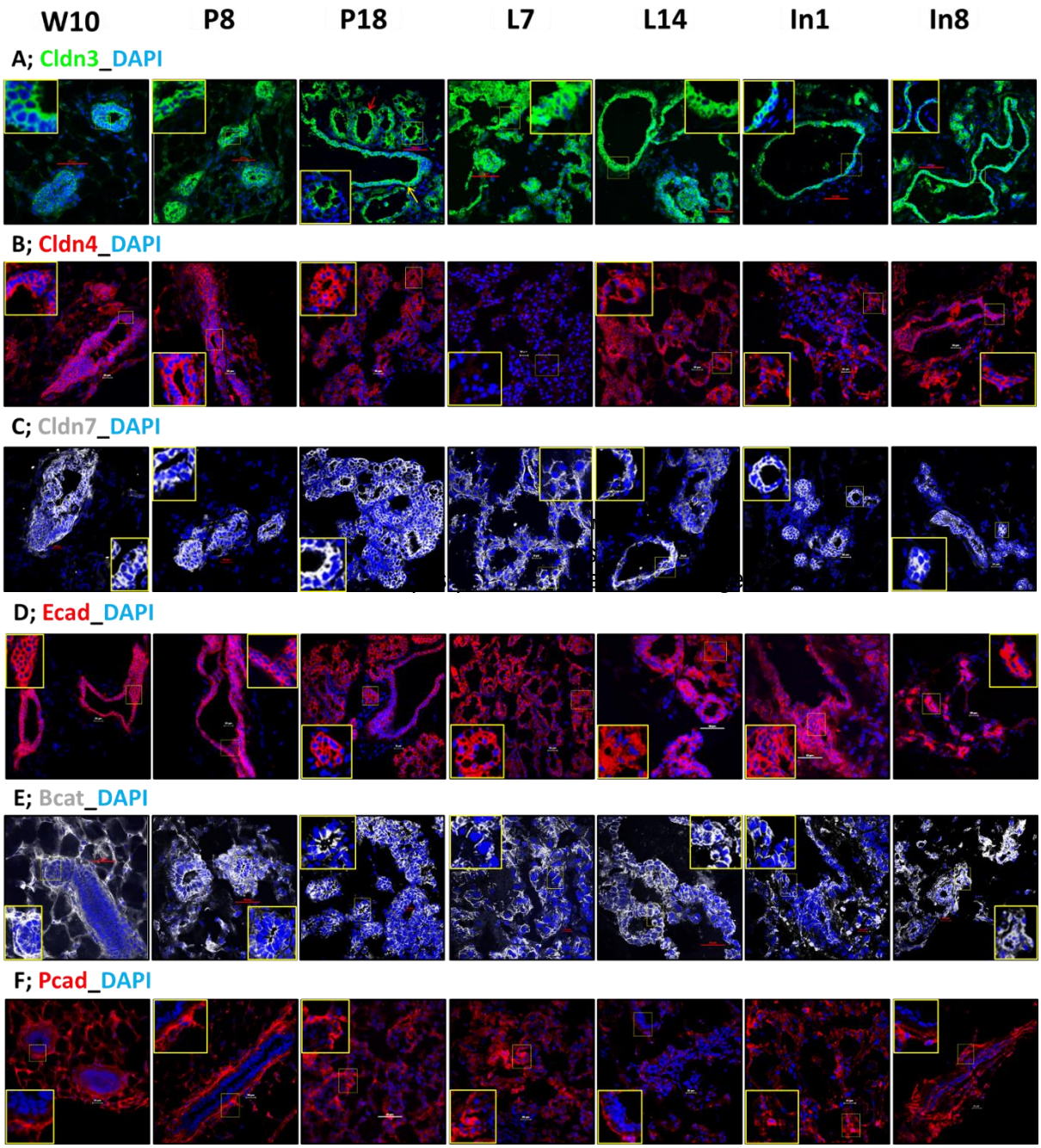

Fig. 9. The localization of claudins, cadherins and β-catenin is modulated throughout different stages of development. Cryosections were cut (7 mm) and processed for immunofluorescent staining. A-C) Cldn-3 (light gray), Cldn-4 (red) and Cldn-7 (light gray) are expressed in luminal cells, but their localization is either apical (red arrow) or basolateral (yellow arrow) during mammary gland development. D) E-cad (red) is detected mainly in luminal cells. E) β-cat (light gray) is observed in both epithelial cell types. F) P-cad (red) is localized at the myoepithelial cells. Nuclei are stained with DAPI. Images were obtained with a Nikon A1Rþ equipped with a spectral detector and analyzed using NIS-elements software.

4.2. Components of the TJs and AJs are expressed in a stage-dependent manner

To determine which components of AJs and TJs could potentially interact with Cxs, we first needed to characterize their expression throughout mammary gland development. We selected Cldn-1, - 3, - 4 and - 7 because they have been reported to be expressed in mammary glands or involved in breast cancer (Blanchard et al., 2006; Blackman et al., 2005; Hewitt et al., 2006; Hoevel et al., 2004; Lanigan et al., 2009). We showed that Cldn-1 mRNA exhibited a stage-dependent differential expression pattern. Cldn-1 mRNA increased after puberty and during pregnancy, de-creased during lactation, and peaked at the beginning of involution followed by a dramatic reduction at In8. Our result is consistent with a microarray analysis of Cldn-1 in mouse mammary gland (Blanchard et al., 2006). Interestingly, at the protein level, Cldn-1 was expressed throughout development without any significant changes among the stages, despite the differential mRNA levels. Similarly, Blanchard et al. (Blanchard et al., 2006) reported positive expression of Cldn-1 in mammary epithelial cells at each stage of post-natal mammary gland development. In their study, Cldn-1 staining was confined to the lateral walls of the epithelial cells in virgin, pregnant, and late involuting glands, while very little Cldn-1 staining was observed at In1 (Blanchard et al., 2006). They also observed basolateral staining of the ductal epithelial cells at day 2 of involution and positive staining in the stromal tissues at day 4 and 10 of involution (Blanchard et al., 2006). Combined with our results, these data suggest that Cldn-1 protein levels are regulated by mechanisms other than transcription and that its localization varies in a stage-dependent manner.

Similar to Cldn-1, Cldn-3 and Cldn-4 transcript levels peaked at In1 and showed high and medium expression levels at P18, respectively. However, low to medium expression was observed at other stages. These results are similar to those previously published (Blanchard et al., 2006), with the exception of L7 and L14, when we observed a down-regulation in Cldn-3 levels, while others showed an increase compared with P18. The same trend was observed at the protein level for both Cldn-3 and –4, with the exception that Cldn-3 protein levels remained high during lactation, despite a sharp decrease at the transcript level, suggesting stabilization of the protein during lactation by transcription-in-dependent mechanisms.

Our results demonstrated that Cldn-7 was highly expressed at both the gene and protein level starting at P18, throughout lactation and In1, and decreased at In8, which is consistent with previous results showing that Cldn-7 is constitutively expressed throughout pregnancy and lactation in normal murine mammary glands (Blackman et al., 2005). However, when comparing pregnant and lactating

mice to virgin mice, (Blackman et al. 2005) did not see any increase when Cldn-7 levels were normalized to keratin 19, which is expressed in luminal cells and also shows in-creased protein expression during pregnancy and lactation These results suggest that the increase in Cldn-7 expression is due to an increase in the number of luminal cells. While we cannot exclude this hypothesis, the stage-dependent expression of the other proteins characterized in our study suggests that other factors are involved.

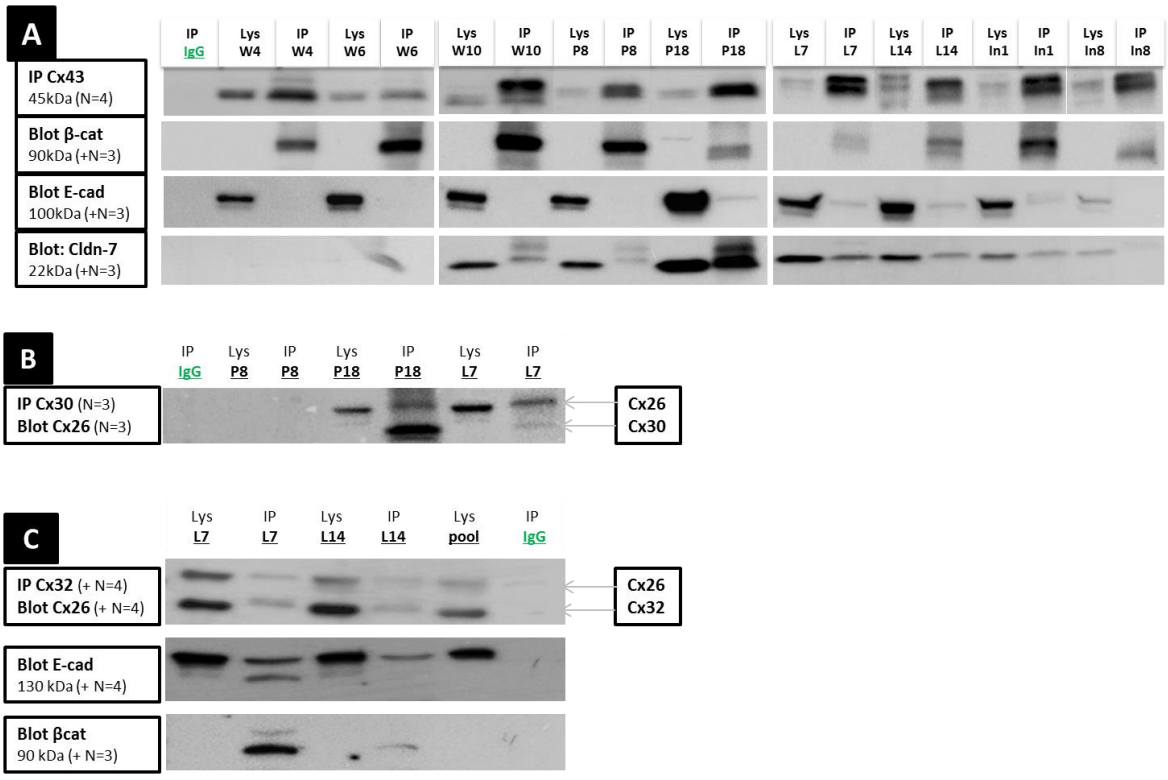

Fig. 10. Connexins interact with components of tight and adherens junctions. A-C) Cx43, Cx30 and Cx32 were immunoprecipitated using total lysates from mammary glands from different developmental stages. Biological replicates were processed at the same time but loaded on different gels when required. A) Cx43 interacts with E-cad, β-cat and Cldn-7 (N= 4). B) Cx30 showed a strong interaction with Cx26 when both are highly expressed at P18 and a weaker interaction at L7 (N=3). C) Cx32 interacts with E-cad, β-cat and Cx26 during lactation (N=4).

Interestingly, when examining the localization of three TJ components, we observed different patterns in the luminal cells along with their different expression profiles. At W10, P8 and during involution, Cldn-3, –4 and 7 demonstrated a basolateral and, to a lesser extent, apical localization in luminal cells. Previous studies also showed positive basolateral staining for Cldn-3 in virgin mice and during pregnancy and late involution, although the staining was more cytoplasmic during lactation

(Blanchard et al., 2006). However, at P18, we observed more apical, rather than basolateral, expression of Cldn-3, -4 and -7. These results suggest that these three claudins mainly provide polarity in the luminal cells that are responsible for milk production. During lactation, Cldn-4 in no longer expressed, while Cldn-3 and −7 re-locate to basolateral sites and intracellular compartments. This transient localization of Cldn-3 and −7 at the apical surface at P18 and their immediate re-localization to the basal and intracellular compartments at L7 suggest that they maintain luminal cell polarity and prevent milk leakage before the onset of lactation. Cldn-7 was previously reported to be located at the basolateral surface of luminal cells in virgin, pregnant and lactating mammary gland in detached vesicles, where it co-localized with the TJ scaffolding protein ZO-1, and in contrast with our results, no apical localization was observed during pregnancy (Blackman et al., 2005).

We then wanted to determine whether Cxs could also interact with AJs by looking at the three main components of AJs: E-cadherin, P-cadherin and β-catenin. While numerous studies have reported the importance of E-cadherin, P-cadherin and β-catenin in cancer initiation, progression and metastasis (Acs et al., 2001; Berx et al., 1995; Daniel et al., 1995), their expression profiles during normal development are not clearly known. At the transcript levels, the three proteins showed similar patterns: their expression increased at puberty, increased during early pregnancy for E-and P-cadherin and late pregnancy for β-catenin, decreased during lactation and increased again during involution. Interestingly, the protein expression profiles were slightly different. E-cadherin and β-catenin both peaked at late pregnancy. E-cadherin then slowly decreased from P18 to In8, remaining high during lactation, whereas β-catenin more rapidly decreased from P18 to In2, remaining high only at L7. In contrast, P-cadherin protein expression peaked at P8, decreased during late pregnancy and early lactation, and then slowly increased until late involution. Again, the high protein expression levels despite low transcript levels at specific stages suggests that the proteins are stabilized through transcription-independent mechanisms. To our knowledge, our study is the first to demonstrate that AJ components are expressed in a stage-dependent manner during mammary gland development.

Our immunofluorescence studies showed that E-cadherin localized at the basolateral cell-cell interface of luminal cells in mammary glands of adult mice and during pregnancy, similar to what has been previously reported for terminal end bud body cells and ductal luminal cells (Daniel et al., 1995). During lactation and at early involution, however, E-cadherin demonstrated both membranous and cytoplasmic localization. β-catenin, which binds to the cytoplasmic tail of cadherins, showed a similar localization in luminal cells but was also expressed in myoepithelial cells and the stroma. This profile is

not surprising because β-catenin is involved in both AJs and signaling pathways (Incassati et al., 2010; Michaelson and Leder, 2001). Our results showed that P-cadherin localized in myoepithelial cells at the plasma membrane in ducts in adult and early pregnancy mammary glands, similar to the observations of others (Daniel et al., 1995). During pregnancy and lactation, myoepithelial cells change shape, changing from a continuous layer of cells to a mesh-like structure (Emerman and Vogl, 1986). Thus, protein localization is harder to observe in myoepithelial cells. P-cadherin seems to be more intracellular in myoepithelial cells and mostly restricted in the stroma at In1 and then gradually re-localizes back to the membrane at In8.

Together, our results demonstrate that Cldn-1 may interact with Cxs at all developmental stages, while Cldn-3 and –7 are more likely able to interact with Cxs in late pregnancy, during lactation and at the beginning of involution. In contrast, Cldn-4 is more likely to interact with Cxs in pubescent, post-pubescent, pregnant and involuting mice. Among the AJ components, E-cadherin is more likely to interact with Cxs from late pregnancy to involution, and β-catenin is more likely to do so during late pregnancy and early lactation. Finally, P-cadherin may interact with Cxs at all stages of development, especially during late pregnancy, involution, and, to a lesser extent, during lactation. Interestingly, most of the junctional proteins that are expressed mainly in luminal cells, namely Cx26, Cx30, Cx32, the claudins and E-cadherin, are highly expressed at late pregnancy and/or lactation, suggesting the formation of a junctional nexus in luminal cells at those stages. In contrast, Cx43 and P-cadherin, which are mainly expressed in myoepithelial cells, were both decreased during lactation. Because some junctional proteins are expressed at the junction between the luminal and myoepithelial cells, junctional protein that are mainly expressed in the luminal cells may interact with some junctional proteins that are mainly ex-pressed in myoepithelial cells.`

4.3. E-cadherin, β-catenin and claudin-7 interact with Cx43 in a phase-dependent manner

Studies in recent decades have demonstrated Cxs interact with a variety of structural and signaling molecules, including cytoskeletal proteins, TJ-associated proteins and AJ-associated proteins (Ai et al., 2000; Giepmans et al., 2001; Angst et al., 1997; Xu et al., 2001; Duffy et al., 2002; Wei et al., 2005; Laird, 2006). Based on our results, Cxs demonstrated a very similar expression pattern to some AJ and TJ components and showed similar localization at the cell membrane. Thus, we determined whether they were inter-acting with these potential binding partners using co-immunoprecipitation analysis. We started by analyzing binding partners of Cx43 because it was present at all stages of development in myoepithelial and luminal cells and at their interface. Cx43 demonstrated a persistent interaction

with β-catenin at all stages tested, although this interaction seemed weaker at the end of pregnancy, during lactation and at late involution, when Cx43 expression is reduced. This interaction between Cx43 and β-catenin was previously reported in the intercalated discs in cardio-myocytes (Ai et al., 2000; Shaw et al., 2007). In cardiomyocytes, mis-localization of these two binding partners is associated with ischemia (Matsushita et al., 1999). Similarly, β-catenin's interaction is necessary for proper positioning of Cx43 at the plasma mem-brane in liver cells (Ale-Agha et al., 2009; Fujimoto et al., 1997). These results suggest that the interaction between Cx43 and β-catenin plays an important role during mammary gland development.

Because β-catenin binds to the cytoplasmic tail of cadherins in AJs, we then investigated the interaction between Cx43 and both E-cadherin and P-cadherin, which are respectively expressed in luminal and myoepithelial cells. Surprisingly, no interaction was found between Cx43 and P-cadherin, suggesting that AJs and GJs do not form junctional nexuses in myoepithelial cells. In contrast, our results showed a phase-dependent interaction between Cx43 and E-cadherin that was stronger during late pregnancy, lactation and early involution. At these stages, E-cadherin was expressed at high levels in the cytoplasm and at the cell membrane of luminal cells, while Cx43 was weakly expressed in luminal cells and abundantly expressed in myoepithelial cells and at the junction of luminal and myoepithelial cells. It has been shown that the interaction between E-cadherin and Cx43 may facilitate the formation and function of GJs in other tissues (Jongen et al., 1991; Her-nandez-Blazquez et al., 2001). Moreover, in a mixed cultures of rat epithelial (BRL) cells and rat (BICR) fibroblasts, E-cadherin expression can enhance GJIC between the two cell types (Prowse et al., 1997). These results suggest that Cx43 can interact with E-cadherin in luminal cells, and/or at the interface of luminal-myoepithelial cells.

It was quite surprising that the interaction between E-cadherin and Cx43 was transient whereas Cx43 and β-catenin interacted at all stages of development. Because β-catenin interacts with the cytoplasmic tail of E-cadherin when it is located in AJs, Cx43 may interact with β-catenin in a cadherin-independent manner at certain stages. β-catenin is involved in signaling pathways, notably the Wnt pathway (Incassati et al., 2010; Michaelson and Leder, 2001). Through its interaction with Cx43, β-catenin may regulate GJIC and cell signaling, with cross-talk with the Wnt signaling pathway (Ai et al., 2000). Whether this cross-talk occurs during mammary gland development remained to be determined.

We next wanted to determine whether the TJ components could interact with Cx43. Our results

showed that an interaction between Cldn-7 and Cx43 is present from adulthood until involution day 1 and that this interaction seems to peak at the end of pregnancy. To our knowledge, this study is the first demonstration of an interaction between Cldn-7 and Cx43. Cldn-7 is expressed in luminal cells, but its localization changes during mammary gland development, shuffling between a basolateral and a more apical localization. Thus, the interaction more probably occurs at the interface of myoepithelial and luminal cells or between the few luminal cells that express Cx43. No interactions were observed be-tween Cx43 and Cldn-3.

Taken together, we observed that Cx43 interacts with components of AJs and TJs in a stage-dependent manner. We cannot conclude whether the interactions between these proteins are direct or indirect, but we presume that Cx43, E-cadherin, β-catenin and Cldn-7 form various junctional nexuses that are required at specific stages. More studies are required to determine the mechanisms and functions linked to the formation, regulation and stability of these nexuses and their exact localization.

4.4. Cx30 interacts with Cx26 but not with AJ or TJ components

Similar to Cx43, Cx30 was immunoprecipitated to identify its binding partners. Cx30 did not show any interaction with the tested AJ and TJ components, suggesting that Cx30 form channels that are not involved in a junctional nexus. Cx30 can form heteromeric and heterotypic channels with Cx26 and Cx32, which are required to support milk production during lactation (Marziano et al., 2003; Locke et al., 2004). We could not detect any interaction between Cx30 and Cx32. Cx30 is expressed at the plasma membrane at P18 and, to a lesser extent, at L7 and L14. In contrast, Cx32 is expressed in intracellular compartments at P18 and is highly expressed at the plasma membrane at L7 and L14, which suggests a switch from Cx30 to Cx32 between those developmental stages.

Our results showed that Cx30 interacts with Cx26 during late pregnancy and early lactation, when they are both highly ex-pressed. These results suggest three non-restrictive possibilities of how Cx30 interacts with Cx26. First, an indirect interaction may occur through an unidentified binding partner, which is likely independent of AJs and TJs. Second, Cx26 and Cx30 may form heterotypic channels. Third, Cx26 and Cx30 may form heteromeric channels. This last hypothesis is supported by the fact that when Cx26 is inhibited, either in the ear or the mammary gland, Cx30 is concomitantly decreased (Stewart et al., 2014; Marziano et al., 2003). While more studies are needed to clarify the interaction between these two Cxs, it does not seem to involve AJs or TJs.

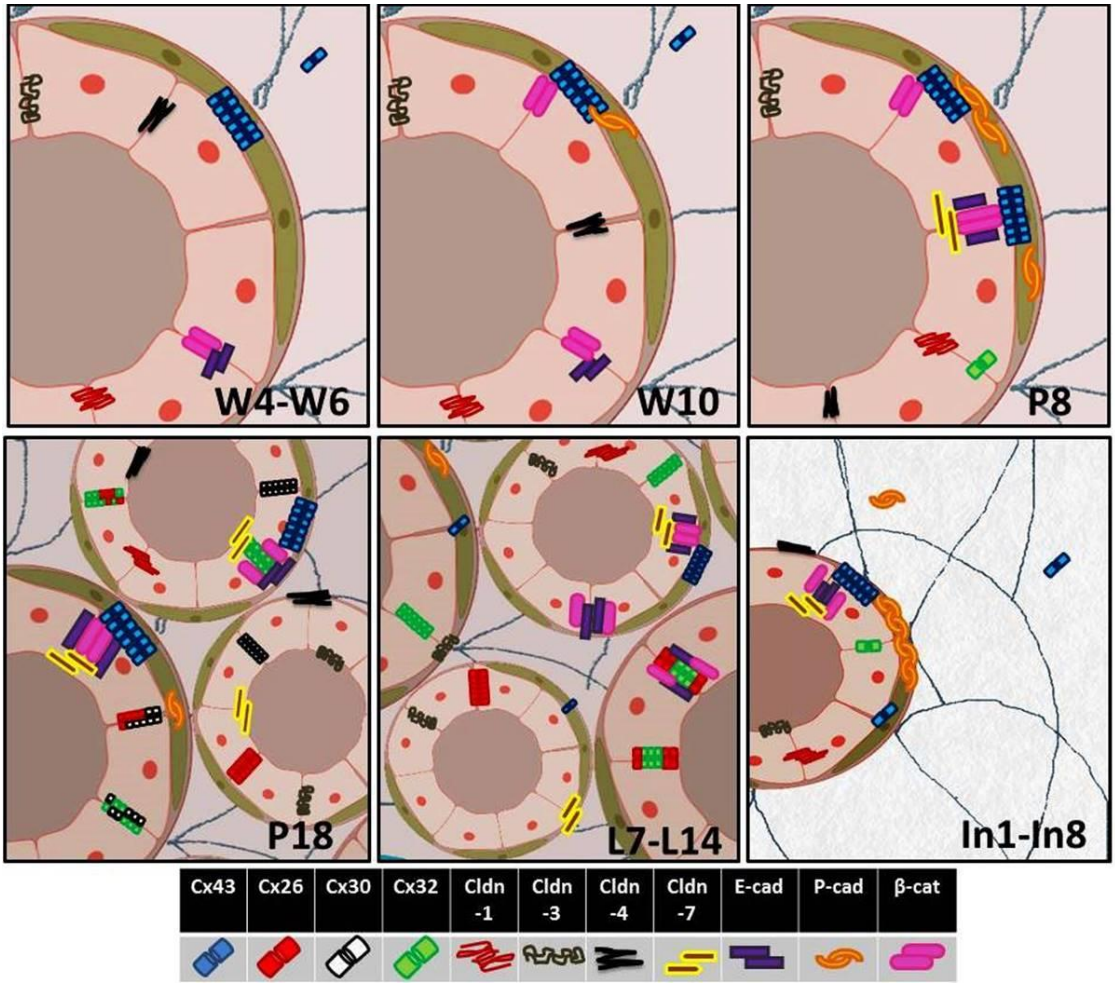

Fig. 11. Proposed model of the junctional nexus found during post-natal mammary gland development. Schematic model of junction protein localization and interactions during mammary gland development.

4.5. Cx32 interacts with Cx26 and AJ components

Cx32 is only expressed at the cell membrane during lactation. Using an immunoprecipitation assay, we first demonstrated that Cx32 interacts with E-cadherin and β-catenin at both tested time point. Although Cx32 co-localization and co-regulation with E-cadherin and β-catenin have been observed in other tissues (Schwarz et al., 2003; Kanczuga-Koda et al., 2014; Ionta et al., 2012; Plante et al., 2005, 2006), to our knowledge, this is the first report of their physical interaction.

Cx32 interacts with occludin, ZO-1 and Cldn-1, which suggests that Cx32 is required for the specific functions of TJs, such as polarity in hepatocytes (Kojima et al., 2001,, 2003,, 1999). Although no interactions were observed with the TJ components tested in our study, we cannot exclude a similar

role for Cx32 in regulating TJs in the mammary gland because not all TJ components were tested.

We also showed that Cx32 interacts with Cx26 during lactation, supporting specific communication between luminal cells during lactation, as previously suggested (Locke et al., 2004). Consistently, Cx26 and Cx32 are also found in the same junctional plaques be-tween epithelial cells and form homomeric and heteromeric channels during lactation (Locke et al., 2000).

Together, our results showed that Cxs interact with AJ and TJs components in Cx-, stage-and localization-dependent manners. These data argue for common regulatory mechanisms that determine the expression of the different junctional proteins and are linked with protein-protein interactions. We thus propose a schematic model of gap, tight and adherens junction protein expression, localization and interaction throughout mammary gland development (Fig. 11). The presence of particular Cxs that interact with junctional nexuses at certain developmental stages suggests that AJs and TJs stabilize proteins at the cell membrane at certain stages and serve stage-dependent roles. Further studies are required to understand the necessity and mechanisms of action for the identified Cx-interacting proteins throughout post-natal mammary gland development.

5. Conclusion

This study allowed us to propose a schematic model describing Gap, Tight and Adherence junctions' protein expression, localization and interaction during mammary gland development (Fig. 11). Presence of particular junctional nexuses at certain stages of development suggests that 1) AJs and TJs may regulate Cx43 and Cx32 functions; 2) AJs and TJs might be implicated in Cxs stabilization at cell membrane at certain stages; and 3) these particular nexuses are required for stage-specific function. Further studies are required to understand the mechanisms involved in Cxs regulation during post-natal mammary gland development.

Conflicts of interest

None to declare.

Acknowledgements

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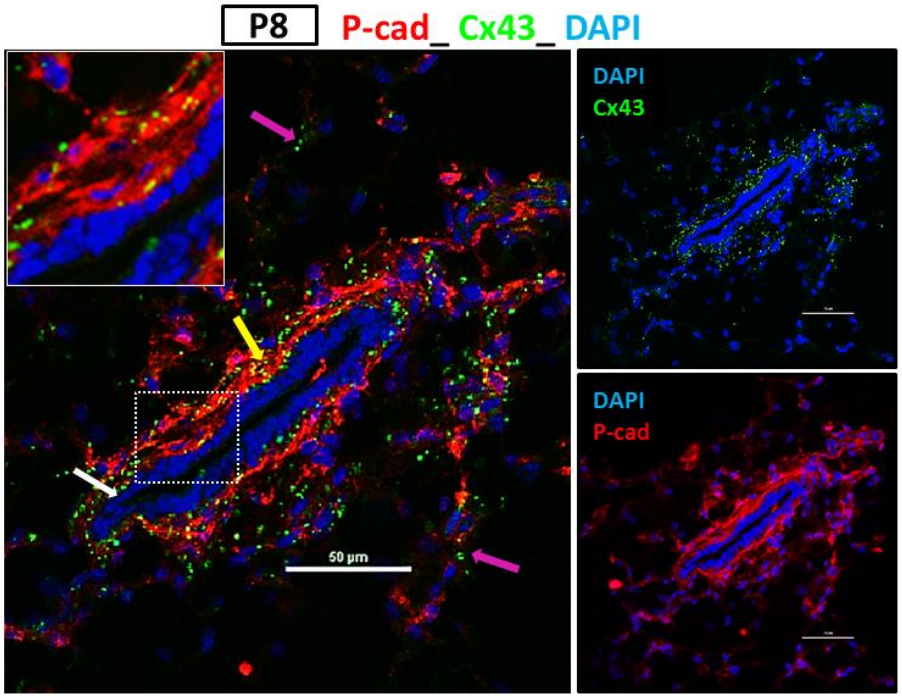
Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.011.

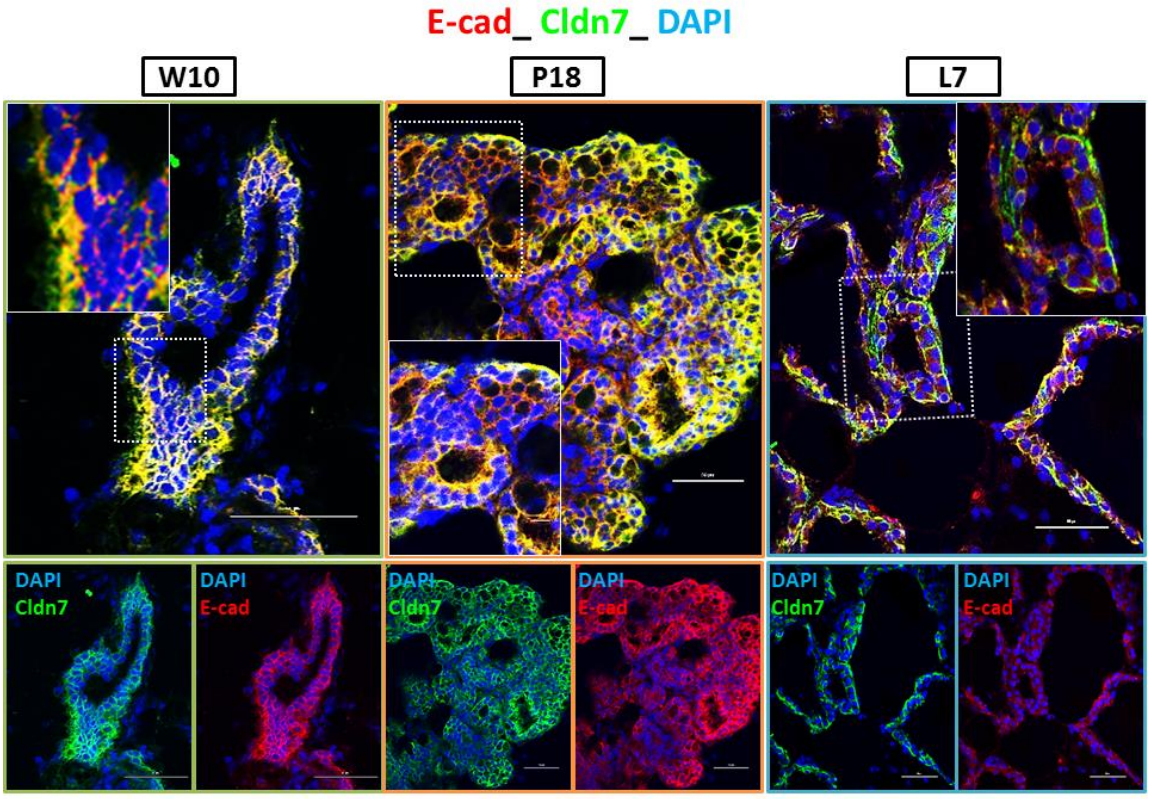
Supplementary table 1) List and sequences of primers used in this study

Name of gene	sequences	Name of gene	sequences
Gapdh_201_F	GAGAGTGTTTCCTCGTCCCG	Ocln_85_F	TGAACTGTGGATTGGCAGC
Gapdh_201_R	ACTGTGCCGTTGAATTTGCC	Ocln_85_R	ATAAGCGAACCTTGGCGGC
Atp5b_239_F	AGCTCTGACTGGTTTGACCG	Cldn2_203_F	CCAGCCTCCAAGGGTTTCAT
Atp5b_239_R	CATAGATAGCCTGCACCGAGG	Cldn2_203_R	TCTAGAAAACGGAGCCGTCC
Cldn1_137_F	TGGGGCTGATCGCAATCTTT	Sdha_151_F	TGCAGGCCTGGAGATAAAGT
Cldn1_137_R	CACTAATGTCGCCAGACCTGA	Sdha_151_R	CTGCAGCATGGTTCTGCATC
Cldn3_103_F	CGTACCGTCACCACTACCAG	Actb_84_F	CCTTCTTGGGTATGGAATCCTG
Cldn3_103_R	CTGTGTGTCGTCTGTCACCA	Actb_84_R	GGTCTTTACGGATGTCAACG
Cldn4_141_F	CCACTCTGTCCACATTGCCT	Hprt_article_F	CCTAAGATGAGCGCAAGTTGAA
Cldn4_141_R	CTTTGCACAGTCCGGGTTTG	Hprt_article_R	CCACAGGACTAGAACACCTGCTAA
Cldn7_88_F	CATTGTGGCAGGTCTTGCTG	RPL13A_article_F	CCTAAGATGAGCGCAAGTTGAA
Cldn7_88_R	CATGGGCGTCAAGGGGTTAT	RPL13A_article_R	CCACAGGACTAGAACACCTGCTAA
Gjb2_87_F	TTCAGACCTGCTCCTTACCG	RPLP0_82_F	GGACCGCCTGGTTCTCCTAT
Gjb2_87_R	GATGGTTGGCACTGTGTCCG	RPLP0_82_R	ACGATGTCACTCCAACGAGG
Cdh1_143(9)_F	TCATCAAATGGGGAAGCGGT	Tbp_146_F	GCAGTGCCCAGCATCACTATTT
Cdh1_143(9)_R	TTCATCACGGAGGTTCCTGG	Tbp_146_R	GTGGAAGGCTGTTGTTCTGG
Cdh3_202_F	CTGCTGACCCTTCTACTGGC	Uxt_197_F	ACTGCAGCGAGACTTGCAAAAG
Cdh3_202_R	CGTTTCGGAGAACCACCTCA	Uxt_197_R	GGGCCACATAGATGCGTGAA
Ctnnb1_137_R	CAGGATGATCCCAGCTACCG		
Ctnnb1_137_R	AGATCAGGCAGCCCATCAAC		
Gja1_173_F	CTTTCATTGGGGGAAAGGCG		
Gja1_173_R	CTGGGCACCTCTCTTTCACTT		
Gjb1_291_F	AGTGCCAGGGAGGTGTGAATG		
Gjb1_291_R	GCTGGGGTGGAAACCAAGATA		
Gjb6_article(288)_F	GGGTACCACCTACCCTGGGTAC		
Gjb6_article(288)_R	TGCATTCTGGCCACTATCTGAG		

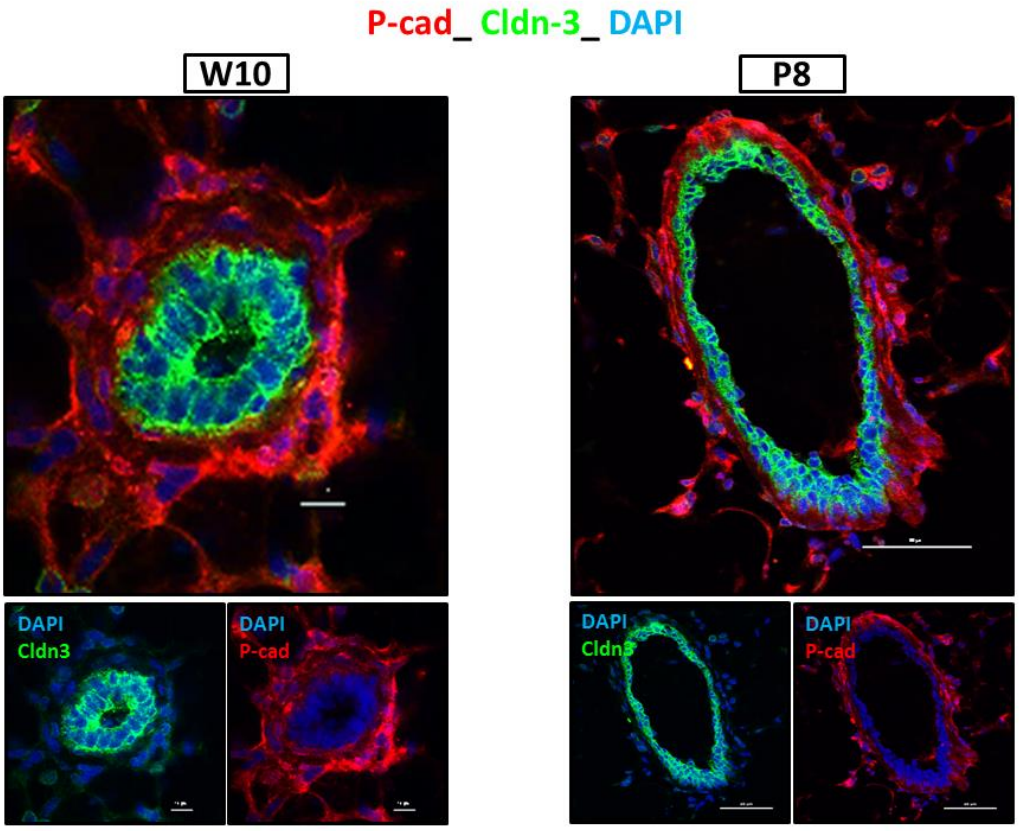
Supplementary figure 1



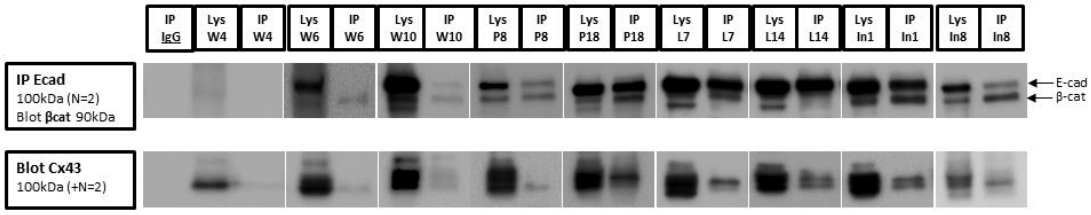
Supplementary figure 2



Supplementary figure 3



Supplementary figure 4



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2.2 Analysis of protein-protein interactions and co-localisation between components of gap, tight and adherens junctions in murine mammary glands

Titre en français: Analyse des interactions protéine-protéine et de co-localisation entre les composants des jonctions gap, serrées et adhérents dans les glandes mammaires murines

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Short Abstract

Intercellular junctions are requisites for mammary gland stage-specific functions and development. This manuscript provides a detailed protocol for studying protein-protein interactions (PPIs) and co-localization using murine mammary glands. These techniques allow for the investigation of the dynamics of the physical association between intercellular junctions at different developmental stages.

Long abstract

Cell-cell interactions play a pivotal role in tissue integrity and the preservation of the barrier between the different compartments of the mammary gland. It is provided by junctional proteins which form nexuses between adjacent cells. Junctional proteins mislocalization and reduced physical associations with their binding partners can result in loss of function and consequently to the organ dysfunction. Thus, identifying proteins localization and protein-protein interactions (PPIs) in normal and disease-related tissues is essential to find new evidences and mechanisms leading to the progress of the diseases or alteration in developmental status. This manuscript presents a two-step method to evaluate PPIs in murine mammary glands. In protocol section 1 a method to perform co-immunofluorescence (co-IF) using antibodies raised against the proteins of interest, followed by secondary antibodies labeled with fluorochromes, is described. Although co-IF allows for the demonstration of the proximity of the proteins, it does make it possible to study their physical interactions. As a result, a detailed protocol for co-immunoprecipitation (co-IP) is provided in protocol section 2. This method is used to determine physical interactions between proteins, without confirming whether these interactions are direct or indirect. In the last few years, co-IF and co-IP techniques allowed to demonstrate that certain components of intercellular junctions co-localize and interact together, creating stage-dependent junctional nexuses that vary during mammary gland development.

Introduction

Mammary gland growth and development occurs mainly after birth. This organ constantly remodels itself throughout the mammal's reproductive life (Sternlicht 2006). The adult mammary gland epithelium is comprised of an inner layer of luminal epithelial cells and an outer layer of basal cells, mainly composed of myoepithelial cells, surrounded by a basement membrane (Oakes, Hilton et al. 2006). For a good review on mammary gland structure and development, the reader can refer to Sternlicht (Sternlicht 2006). Cell-cell interactions via Gap (GJ), Tight (TJ) and Adherens (AJ) junctions are

necessary for the normal development and function of the gland (El-Sabban, Abi-Mosleh et al. 2003, Gudjonsson, Adriance et al. 2005, Hennighausen and Robinson 2005, Stein, Hilton et al. 2006, Sternlicht 2006). The main components of these junctions in the murine mammary gland are Cx26, Cx30, Cx32 and Cx43 (GJ), Claudin-1, -3, -4, -7 and ZO-1 (TJ) and E-cadherin, P-cadherin and β-catenin (AJ) (Nguyen and Neville 1998, Stewart, Simek et al. 2015). The levels of expression of these different junctional proteins vary in a stage-dependant manner during mammary gland development, suggesting differential cell-cell interactions requirements (Dianati, Poiraud et al. 2016). GJ, TJ and AJ are linked structurally and functionally, and tether other structural or regulatory proteins to the neighboring sites of adjacent cells, thus creating a junctional nexus (Derangeon, Spray et al. 2009). The composition of the junctional nexus can impact bridging with the underlying cytoskeleton as well as nexus permeability and stability, and consequently influence the function of the gland (Derangeon, Spray et al. 2009)' (Hernandez-Blazquez, Joazeiro et al. 2001). The components of intercellular junctions residing in junctional nexuses or interacting with one another at different developmental stages of mammary gland development were analyzed recently using co-IF and co-IP (Dianati, Poiraud et al. 2016). While other techniques allow for evaluation of functional association between proteins, these methods are not presented in this manuscript.

As proteins merely act alone to function, studying protein-protein interactions (PPIs) is essential for many research, such as signal transductions and biochemical cascades, and can provide significant information about the function of proteins. Co-IF and microscopy analysis help to evaluate a few proteins sharing the same subcellular space; however, the number of targets is limited by the antibodies needing to be raised in different animals and the access to a confocal microscope equipped with different wavelength lasers and spectral detector for multiplexing. Co-IP confirms or reveals high-affinity physical interactions between two or more proteins residing within a protein complex. Despite the development of novel techniques such as Fluorescence Resonance Energy Transfer (FRET) (Kiyokawa, Hara et al. 2006) and Proximity Ligation Assay (PLA) (Gustafsdottir, Schallmeiner et al. 2005), which can detect the localization and interactions of proteins simultaneously, co-IP remains an appropriate and affordable technique to study interactions between endogenous proteins.

The step-by-step method described in this manuscript will facilitate the study of protein localization and PPIs, and point out pitfalls to avoid when studying endogenous PPIs in the mammary glands. The methodology starts with a presentation of the different preservation procedures for tissues

required for each technique. Then, part 1 presents how to study proteins co-localization in three steps: i) Sectioning of mammary glands; ii) Double or triple labeling of different proteins using the co-IF technique; and iii) Imaging of the localization of proteins. Part 2 shows how to precipitate an endogenous protein and identify its interacting proteins in three steps: i) Lysates preparation; ii) Indirect protein immunoprecipitation; and iii) Identification of binding partners by SDS-PAGE and Western blot. Each step of this protocol is optimized for rodent mammary gland tissues and generates high quality, specific and reproducible results. This protocol can also be used as a starting point to whoever wants to study PPIs in other tissues or cell lines.

Protocol

All animal protocols used in this study were approved by the University Animal Care Committee (INRS-Institut Armand-Frappier, Laval, Canada).

1. Identifying proteins co-localization

1.1. From tissue to microscopic slides

Note: Tissues and sections should be handled on dry ice.

1.1.1. Excise mammary glands from the animal (for a complete description of this procedure, refer to (Plante, Stewart et al. 2011).

1.1.2. Embed excised tissue in freezing/mounting media on dry ice. Add enough media to cover the gland. When the media is solidified, transfer the tissues to a freezer at -80 °C for later use9.

1.1.3. Using a cryomicrotome set at ≤-35 °C, cut the tissues into sections of 7-10 µm and place them on microscope slides.

Note: When possible, place two sections on each slide; the left section will be used as a negative control to verify the specificity of the antibodies used and autofluorescence of the tissue, while the right side will be labeled with the antibodies.

1.1.4. Keep sections at -80 °C for later use.

1.2. Co-Immunofluorescence staining

1.2.1. Retrieve the appropriate microscopic slides from the freezer and immediately fix the sections by immersing them in formaldehyde 4% for 15 min at room temperature (RT).

1.2.2. Then immerse the slides in phosphate-buffered saline (PBS) at RT. Leave the slides in phosphate-buffered saline (PBS) at RT until proceeding to the next step.

1.2.3. Circle each section on the slide using a commercially available hydrophobic barrier or water repellent lab pen (see Table of material). Be careful not to touch the tissue. Then, immediately add drops of PBS on the tissue and place the slide in a humid histology chamber for the remainder of the procedure.

Note: Tissue sections must remain moisturized. Alternatively, use a box with a lid and place wet paper towels on the bottom.

1.2.4. Block each tissue section with 100-200 µL of 3% bovine serum albumin (BSA)-tris-buffered saline (TBS)-0.1% polysorbate 20 (see Table of material) for 30 min at RT. While samples are blocking, prepare the primary and secondary antibody solutions by diluting the antibodies in TBS-0.1% polysorbate 20.

Note: Required concentration for the antibody is provided by manufacturer; see Table of materials and figures 1-2 for examples, as well as Dianati et al.[9]. Although it is not necessary to work in the dark when using most fluorophore conjugated antibodies, avoid exposing the antibody solutions or stained tissues to the intense bright light.

1.2.5. Remove the blocking solution by aspiration and incubate the sections with 100-200 µL of the diluted primary antibody for 60 min at RT. Alternatively, incubate with the primary antibody overnight at 4 °C.

1.2.6. Remove the primary antibody solution by aspiration and wash sections with 250-500 µL of TBS-0.1% polysorbate 20 for 5 min. Remove the wash solution by aspiration and repeat the washes twice.

1.2.7. Remove the wash solution by aspiration and incubate sections with 100-200 µL of the appropriate fluorophore conjugated secondary antibody for 60 min at RT.

1.2.8. Remove the secondary antibody solution by aspiration and wash sections with 250-500 µL of TBS-0.1% polysorbate 20 for 5 min. Remove the wash solution and repeat twice.

1.2.9. Repeat step 2.5-2.8 using the appropriate combination of first and secondary antibodies for the subsequent protein(s) of interest.

1.2.10. Remove the wash solution by aspiration and perform the nuclei staining by incubating the section with 100-200 µL of 1 mg/mL of 4',6-diamidino-2-phenylindole (DAPI) in TBS-0.1% polysorbate 20 for 5 min at RT.

1.2.11. Remove the DAPI solution by aspiration and mount the slides using a water-soluble, non-fluorescing mounting medium (see Table of material) and coverslips. Proceed one slide at a time.

Note: Incubation of nuclei stain for more than 5 min would not change the intensity of the staining. Alternatively, remove the DAPI solution on all slides and incubate the tissues in PBS while mounting the slides.

1.2.12. Place slides lying flat in a 4 °C refrigerator for at least 8 h. Then proceed to perform fluorescence microscopic imaging (See Figures 1, 2).

1.3. Microscopic imaging

1.3.1. Visualize the fluorophore conjugated secondary antibodies using a confocal microscopy equipped with the various lasers required to excite the fluorophores at their specific wavelengths. Note: To be able to visualize ducts and alveoli, a 40X objective with a numerical aperture of 0.95 is suggested.

Note: An example of specific settings is provided in Figure 1.

1.3.2. Verify the localization of each protein individually by scanning the image one wavelength at the time.

Note: At this stage, it is important to critically analyze the localization of junctional proteins. To be able to form junctional nexuses, these proteins have to be localized at the plasma membrane.

1.3.3. Determine co-localization of proteins by merging images scanned with the lasers at the different wavelengths.

Note: Proteins co-localization can be visualized by change of color resulting from the emission of two or more fluorophores at the same location, and measured using appropriate software (Figures 1-2; see also 9).

2. Studying protein-protein interactions (PPIs)

Note: For studying PPIs, abdominal mammary glands should be used, as thoracic glands are in close association with pectoral muscles. Excise mammary glands (for a complete description of this procedure, refer to Plante et al.[14]) and keep the glands at -80 °C for later use.

2.1. Lysate preparation

2.1.1. Place weighing paper and 2 mL microcentrifuge tubes on dry ice to pre-cool them before proceeding with the next steps.

2.1.2. Take the mammary gland tissue from the -80 °C and keep them on dry ice.

2.1.3. Weigh the tissues on the pre-chilled weighing papers; then transfer the tissue to 2 mL microcentrifuge tubes (handle on dry ice). Use between 50-100 mg of tissue per sample. Keep the tissue on dry ice until step 2.1.5.

2.1.4. Prepare the required amount of triple detergent lysis buffer supplemented with NaF, NaVO3 and protease/phosphatase inhibitor, as indicated in Table of material, using the following formulas. Mice: required buffer (µL) = mice tissue weight (mg) x3; Rat: required buffer (µL) = rat tissue weight (mg) x5.

2.1.5. Add the required amount of ice cold lysis buffer (calculated at step 2.1.4) to the 2 mL tube containing the tissue.

Note: In steps 2.1.5-2.1.6, proceed with one single tube at a time.

2.1.6. Homogenize the tissue for 30-40 s using continuous homogenization on a tissue grinder; always keep the tube on ice. Adjust the tissue homogenizer to medium speed and gently move the grinder up and down inside the tube.

2.1.7. Repeat steps 1.6 and 1.7 with the other tubes.

2.1.8. Incubate the lysates on ice for 10-30 min.

2.1.9. Centrifuge the tubes at 170 x g for 10 min at 4 °C.

2.1.10. Meanwhile, identify 6-10 microcentrifuge tubes (0.6 mL) for each sample, and keep them on ice.

2.1.11. Once the centrifugation is done, check the tubes. Ensure that they contain a top layer of fat, clear yellow to pink lysates (depending on the stage of development) and a pellet.

2.1.12. Create a hole in the lipid layer using a 200 µL pipette tip to access the liquid phase. Change the tip and collect the lysate without disturbing the pellet or aspirating the lipid layer. Aliquot the lysate in pre-labeled tubes on ice (step 2.1.11) and store them at -80 °C.

2.1.13. Use an aliquot to quantify the protein concentration using an appropriate commercially available kit (see Table of materials).

2.2. Indirect immunoprecipitation

2.2.1. Thaw two aliquots of the total mammary gland lysates prepared previously on ice.

Note: one aliquot will be used for the IP of the target protein, while the other will serve as negative control.

2.2.2. Collect 500-1000 µg of the lysate and dilute it in PBS to reach a final volume of 200 µL in each 1.5 mL tube.

Note: The amount of lysate to be used depends on the abundance of the protein of interest, and the efficiency of the antibody (see figure 3 for an example, and Table of materials). It should be optimized for each target by using different amount of lysate (i.e. 500; 750; 1000 µg) and of antibody (i.e. 5; 10; 20 µg), and proceeding with the following steps (2.2.3-2.3.7.4).

2.2.3. Add the antibody against the antigen of interest to the first tube of lysate and keep it on ice.

Note: The required amount is usually suggested on the instruction sheet provided by each company (see Table of material).

2.2.4. Prepare a negative control by adding the same concentration of isotype IgG control as the antibody used in step 2.2.3 in the second tube.

2.2.5. Incubate the tubes overnight at 4 °C on a tubes roller-mixer at low speed.

2.2.6. The following day, add 50 µL of magnetic beads to new 1.5 mL tubes for pre-washing.

2.2.6.1. Select either Protein A or Protein G magnetic beads based on the relative affinity to the antibody.

2.2.6.2. It is important to avoid using aggregated beads; gently mix the bead suspension until it is uniformly re-suspended before adding it to the tubes.

2.2.7. Place the tubes containing the beads on the magnetic stand and allow the beads to migrate to the magnet. Then, remove the storage buffer from the beads using a 200 µL pipette.

2.2.8. Wash the beads by adding 500 µL of PBS-0.1% polysorbate 20 and vortex the tubes vigorously for 10 s.

2.2.9. Put the tubes back on to the magnetic stand and allow the beads to migrate to the magnet.

2.2.10. Remove the excess wash buffer by pipetting using 200 µL pipette.

2.2.11. Add the reaction complex (lysate-antibody) from step 2.2.5 to the beads and incubate this for 90 min at RT on the roller mixer.

2.2.12. Place the tubes on the magnetic stand and allow the beads to migrate to the magnet. Using a 200 µL pipette aspirate and discard the lysate and place the tubes on ice.

2.2.13. Wash the beads by adding 500 µL of PBS to the beads, place the tubes on the magnetic stand and remove the liquid using a 200 µL pipette. Repeat this wash step. During the wash steps avoid vortexing and keep samples on ice.

2.2.14. Wash the beads once with PBS-0.1% polysorbate 20 without vortexing, and discard the last wash buffer using a 200 µL pipette tip.

2.2.15. To elute, add 20 µL of 0.2 M acidic glycine (pH = 2.5) to the tubes and shake them for 7 min on the roller mixer.

2.2.16. Centrifuge at high speed for a few seconds (quick spin), and collect the supernatant in a fresh ice-cold tube.

2.2.17. Repeat steps 2.2.14 and 2.2.15 for each tube.

Note: The final volume will be 40 µL.

2.2.18. Add 10 µL of 4x laemmli buffer to the 40 µL eluted sample from step 2.2.16.

Note: The color turns yellow due to acidic pH.

2.2.19. Immediately add 1 M tris (pH = 8), one drop at a time, to the eluted sample from step 2.2.18 until its color turns blue. Proceed to the next tubes.

2.2.20. Boil the samples from step 2.2.18 at 70-90 °C for 10 min. Proceed immediately to gel electrophoresis. Alternatively, transfer the samples to a freezer at -80 °C until loading.

2.3. Downstream application: gel electrophoresis followed by western blot

2.3.1. Prepare separating and stacking SDS-PAGE acrylamide gels (1.5 mm thickness) following standard procedures[15].

Note: The choice of gel (8-15% acrylamide, gradient: see Table of material) should be determined based on the molecular size of the protein to be precipitated and of the potential binding partners to be analyzed. These proteins have to be resolved from each other to allow proper immunodetection.

2.3.2. Thaw the immunoprecipitation (IP) eluted samples (step 2.2.20) on ice.

2.3.3. Prepare protein lysates from the same samples (used for the IP procedure above). Use 50 µg of total lysate and add 4X Laemmli sample buffer. Boil samples at 70-90 °C for 5 min and place on ice until loading.

Note: These samples will be loaded beside the eluted IP sample to demonstrate the presence of precipitated proteins in total lysate.

2.3.4. Load the prepared lysates (from step 2.3.3) and the precipitated samples from step 2.2.20 side by side in acrylamide gel and run them in running buffer (10x running buffer: Tris 30.3 g/glycine 144.1 g /SDS 10 g in 1 L distilled water) at 100V for approximatively 95 min, or until the edge of the migrating proteins reach the bottom of the gels.

2.3.5. Transfer the gels to a nitrocellulose or PVDF membrane, using a standard protocol [9,15].

2.3.6. Block the membrane for 1 h on a rocker on low speed in 5% dry milk-TTBS (20 mM Tris, 500 mM NaCl, 0.05% polysorbate 20).

2.3.7. Identify whether the precipitation was successful.

2.3.7.1. Probe membrane using the first antibody against the precipitated protein, diluted in 5% dry-milk-TTBS at the concentration recommended by the manufacturer, overnight at 4 °C on a rocking platform with slow agitation.

Note: See Table of material for recommendations.

2.3.7.2. The following day, wash the membrane 3 times for 5 min with TTBS on a rocking platform with high agitation.

2.3.7.3. Incubate the membrane in the appropriate secondary antibody conjugated with horseradish peroxidase (HRP), diluted in TTBS, for 1 h at RT on a rocking platform with slow agitation.

Note: Alternatively, a secondary antibody conjugated with a fluorochrome can be used if an appropriate apparatus to detect the signal is available.

2.3.7.4. Perform 3 to 6 washes, each for 5 min with TTBS on a rocking platform with high agitation. Analyze the signal of the secondary antibody by incubating the membrane with a commercially

available luminol solution (see Table of material) and follow the manufacturer instructions. Detect the signal using a chemiluminescence imaging system (see Table of material).

Note: For a detailed protocol on western blot analysis, see16.

2.3.8. To identify interacting protein, perform steps from 2.3.7.1 to 2.3.7.4 using the appropriate antibodies on the same blot.

Note: If proteins are interacting, the binding partners will be co-immunoprecipitated with the target protein, and thus be detectable by western blotting. Step 2.3.8 can be repeated with more antibodies to determine whether other proteins reside in the same proteins complex, as long as the molecular weight of the proteins differs enough to be well-separated on the gel and membrane.

2.3.9. To confirm that the identified binding partners are not artifacts, reciprocal IP should be performed.

Note: This is performed by repeating steps 2.2.1-2.2.20 with the same lysate, but by precipitating one of the binding partners identified in step 3.8. Then, steps 3.1-3.8 are repeated using the primary antibody against the first protein of interest.

Representative results

In order to determine whether GJ, AJ and TJ components can interact together in the mammary gland, co-IF assays were first performed. Cx26, a GJ protein, and β-Catenin, an AJ protein, were probed with specific antibodies, and revealed using a fluorophore conjugated mouse-647 (green, pseudocolor) and goat-568 antibodies (red), respectively (Figure 1B, 1C). Data showed that they co-localize at the cell membrane of epithelial cells in the mice mammary gland at lactation day 7 (L7), as reflected by the yellow color (Figure 1D). Secondly, Claudin-7, a TJ protein, E-cadherin, an AJ protein, and Connexin26 (Cx26), a GJ protein, were probed with their specific antibodies and revealed with a fluorophore conjugated rabbit-488 (green), mouse-555 (red) and mouse-647 (coral blue; pseudocolor) antibodies, respectively (Figure 2B-D). E-cadherin and Claudin-7 co-localization is displayed as a yellow-to-light-orange color, while Cx26 co-localization with E-cadherin and Claudin-7 resulted in white punctuated staining in mice mammary gland at pregnancy day 18 (P18) (Figure 2E).

In order to find out which junctional proteins intermingle and physically tether together at the cell membrane, co-immunoprecipitation was performed using mammary gland tissues from lactating mice (L14). Results showed that Cx43, a component of GJ, interacts with E-Cadherin and Claudin-7, but not

with Claudin-3 (Figure 3A, B). These results were confirmed by the reciprocal IP; when E-Cadherin was immunoprecipitated, it interacted with Cx43 and Claudin-7 (Figure 3C).

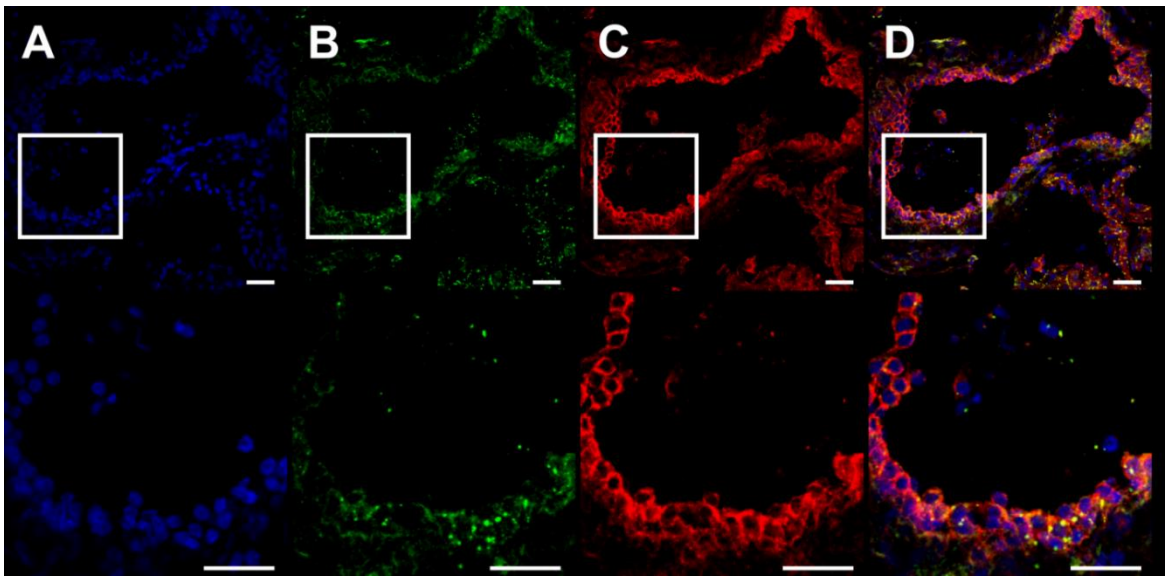

Figure 1. β-Catenin and Cx26 co-localize at the cell membrane in mice mammary glands. Cryosections from mammary glands of mice at lactation day 7 (L7) were cut (7 µm) and processed for immunofluorescent staining. (**A**) Nuclei were stained with DAPI (blue). (**B**) Cx26 (green, pseudocolor) and β-Catenin (**C**) (red) are shown combined with appropriate fluorophore-labeled antibodies. (**D**) Shows a merged image. Images were obtained with a confocal microscope equipped with a spectral detector. DAPI was visualized using the following settings: Emission wavelength: 450.0 nm; Excitation wavelength: 402.9 nm; Laser power: 1.2; Detector gain: PMT HV 100, PMT offset 0. Cx26 (647) was visualized using the following settings: Emission wavelength: 700.0 nm; Excitation wavelength: 637.8 nm; Laser power: 2.1; Detector gain: PMT HV 110, PMT Offset 0. β-Catenin (568) was visualized using the following settings: emission wavelength: 595.0 nm; excitation wavelength: 561.6 nm; Laser power: 2.1; Detector gain: PMT HV 110, PMT offset 0. Scale bars: 50 µm

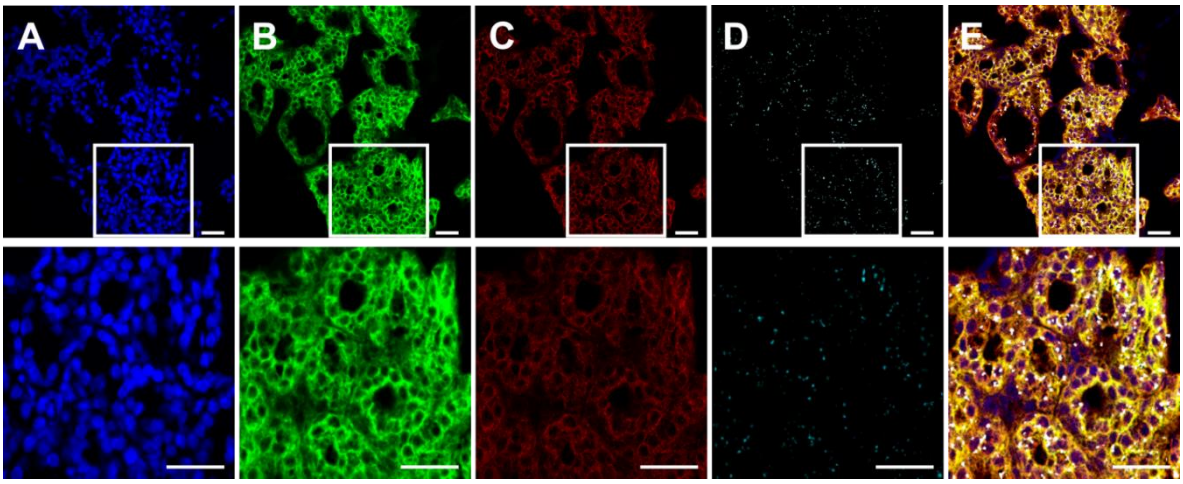

Figure 2) Connexin26 (Cx26), E-cadherin, Claudin-7 co-localize at the cell membrane in mice mammary glands. Cryosections from mammary glands at pregnancy day 18 (P18) were cut (7 µm) and processed for immunofluorescent staining using (**B**) Claudin-7 (green), (**C**) E-Cadherin (red) and (**D**) Cx26 (coral blue; pseudocolor) combined with appropriate fluorophore-labeled antibodies. (**A)** Nuclei were stained with DAPI (blue). Images were obtained with a confocal microscope equipped with a spectral detector. DAPI was visualized using the following settings: Emission wavelength: 450.0 nm; Excitation wavelength: 402.9 nm; Laser power: 5.4; Detector gain: PMT HV 65, PMT offset 0. Claudin-7 (488) was visualized using the following settings: Emission wavelength: 525.0 nm; Excitation wavelength: 489.1 nm; Laser power: 5.0; Detector gain: PMT HV 12, PMT Offset 0. E-Cadherin (568) was visualized using the following settings: emission wavelength: 595.0 nm; excitation wavelength: 561.6 nm; Laser power: 13.5; Detector gain: PMT HV 45, PMT offset 0. Cx26 (647) was visualized using the following settings: emission wavelength: 700.0; excitation wavelength: 637.8; Laser power: 5.0; Detector gain: PMT HV 55, PMT Offset 0. Scale bars: 50 µm

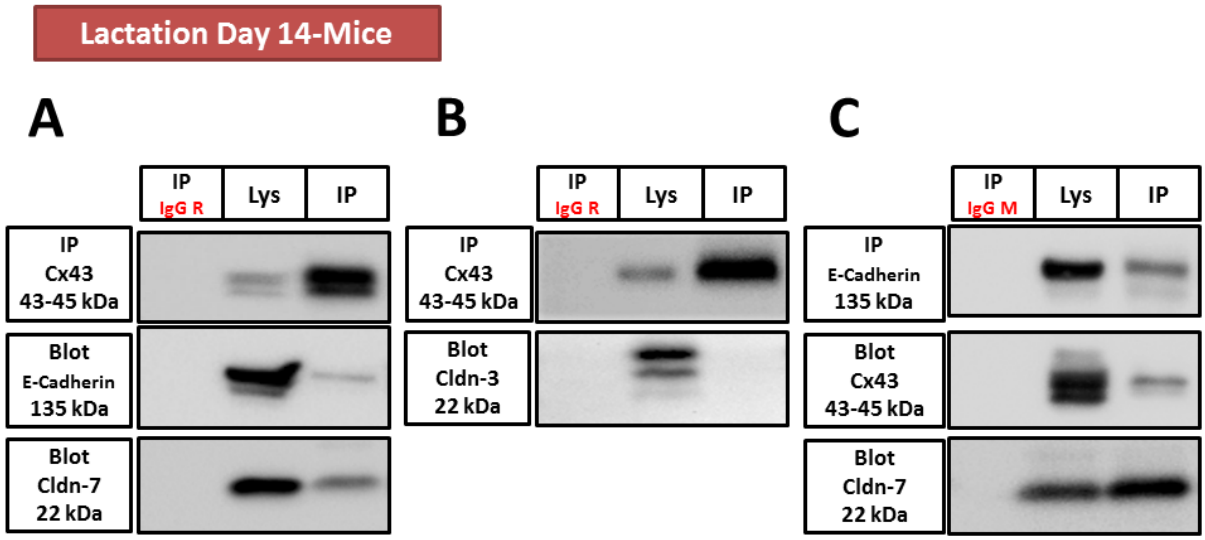

Figure 3) Cx43, E-Cadherin and Claudin-7, but not Claudin-3, are involved in a protein complex. Cx43 (**A, B**) and E-Cadherin (**C**) were immunoprecipitated using 500 mg of total lysates from mammary glands of mice at lactation. IPs and lysates were loaded in gels and transferred on PVDF membranes. Because Claudin-7 and Claudin-3 have the same molecular weight, they could not be analyzed on the same membrane. Thus, two parallel IP were performed with the same lysate for Cx43, loaded on two gels and then transfer (membranes A and B). Membrane A was first probed with Cx43 to confirm the efficiency of the IP (top panel). Then the membrane, was sequentially probed with E-Cadherin and Claudin-7. Western blot analysis showed that the two proteins interact with Cx43. Membrane B was first probed with Cx43 to confirm the efficiency of the IP (top panel), and then probe with Claudin-3. Western blot analysis showed that Claudin-3 did not IP with Cx43, demonstrating that the two proteins are not interacting. Membrane C was first probed with E-cadherin to confirm the efficiency of the IP (top panel). Then the membrane, was sequentially probed with Cx43 and Claudin-7. Western blot analysis confirmed the interactions between the proteins.

Discussion

Cell-cell interactions via junctions are required for proper function and development of many organs such as the mammary gland. Studies have shown that junctional proteins can regulate one another's function and stability, and activate signal transduction by tethering each other at the cell membrane (Derangeon, Spray et al. 2009). The protocols presented in the current manuscript has provided interesting findings about junctional proteins differential expression, localization and interaction during normal murine gland development (Dianati, Poiraud et al. 2016). Given that the localization of junctional proteins' is critical for the function of the proteins, and because they are known to interact with scaffolding proteins and numerous kinases (Stein, Hilton et al. 2006), co-IF and co-IP are effective techniques in the field of cell-cell interactions. Not only are these methods essential

to enlighten the necessity of junctional nexuses in mammary gland development and their dysregulation in breast cancer, but they can also be used in other tissues, and in experiments using cell lines.

The mammary gland is composed of two main compartments: the stroma and epithelium[4]. The adult epithelium is made of two layers of cells. This approach was used to determine the proximity of the potential binding partners using the co-IF technique, and to confirm their physical interaction using co-IP. Co-IF has been successfully used by others to demonstrate the co-localization of proteins within the same tissue, structure, cell or intracellular compartment (Oxford, Everitt et al. 2007) (Wang and Li 2014). The main advantage of this technique is the visual information it brings about the cellular or subcellular localization of each protein within the different cell types composing the tissue. Although this technique is quite simple, some recommendations must be followed. By instance, to avoid tissues damage, always add the solutions one drop at a time using 200 µL pipettes, or a transfer pipette. This will allow the immersion of the tissue surface without damaging the tissues. Similarly, remove the solutions using a Pasteur pipette placed beside the tissues by gently tilting the slide. Moreover, for antibodies whose storage solution contains glycerol, careful suction of the wash buffers is required to reduce background. Moreover, the presence of milk proteins, such as caseins, during lactation can interfere with the antibodies by trapping them, resulting in false positive. A critical analysis of the resulting image is thus required, specifically at this stage.

This co-IF technique also has certain limitations or pitfalls. First, it requires specific antibodies. As mentioned previously (1.1), it is recommended to use one section (i.e. the one on the right side) on each slide for staining following the steps described above, adding the primary and secondary antibodies sequentially. For the remaining section (i.e. the one on the left side), follow the same procedure, using TBS-Polysorbate 20 0.1% instead of primary antibodies solutions. While it is also possible, and even better, to verify the specific binding of the antibody using peptide competition, peptides used to generate commercial antibodies are not always available. It is thus important to verify the specificity of the binding using positive and negative controls (i.e. tissues or cells known to express, or not, the protein of interest). Moreover, antigen fixation sites can be inaccessible, especially for formalin-fixed tissues, thus resulting in absence of a specific signal. An antigen-retrieval procedure may thus be required for some tissues. A short incubation with a detergent can also be performed prior to antigen-retrieval to permeabilize cells membrane. Second, for multiplexing, antibodies have to be

raised in different animals. For instance, if anti-rabbit were used in step 1.2.6, anti-mouse can be selected in step 1.2.10, but not another antibody raised in rabbit. Since most commercial antibodies are raised in rabbits, mice or goats, it is sometimes difficult, or even impossible, to target two proteins at the same time due to the lack of appropriate antibodies. To overcome these limitations, one can either buy commercially available pre-labeled antibodies or label primary antibodies with fluorophores using commercially available kits. Third, another shortcoming is linked to the excitation and emission of the different fluorophores. To avoid overlapping of the signals from two different antibodies, the excitation-emission spectrum of each fluorophore needs to be separated. Thus, the number of targets that can be analyzed at once will vary according to the configuration of the available microscope. Finally, the quality of the analysis is highly dependent on the microscope used. More detailed and precise data can be obtained using a confocal microscope compared to an epifluorescent microscope. The use of super-resolution microscopy can reveal proteins co-localization with even more details (Bertocchi, Wang et al. 2017).

Although co-IF brings important insights about the proximity of potential binding partners, it should be complemented by other methods to identify physical interactions between proteins. Among methods available, co-IP is probably one of the most affordable to perform as the equipment and material are easily accessible. Using an antibody bound to magnetic beads, one can isolate protein complexes and identify the components present in that complex using typical western blot analysis. Similar to co-IF, some recommendations should be followed for best practices. For instance, it is recommended to minimize the samples when homogenizing the tissues to reduce the time between steps 2.1.5 and 2.1.9. While an experienced person can process up to 10 samples at a time, beginner should not handle more than 4-6 samples. Similarly, the number of tubes should be limited when performing the IP protocol for the first time. It is recommended to start with a negative control (i.e. IgG) and a positive control (i.e. a tissue known to contain the protein to be immunoprecipitated) only. The second trial should be dedicated to optimization (see step 2.2.2). Once these steps gave satisfying results, samples to be analyzed can be processed. Note that a negative control should always be included in the procedure.

This co-IP technique also has potential pitfalls. First, it requires tissues to be homogenized in conditions permitting the preservation of the links between proteins. For membrane proteins such as junctional proteins, it is also crucial to use a buffer that will preserve the bounds between the proteins

while allowing their solubility. Second, similar to co-IF, it requires high-affinity antibodies for both the target protein and binding partners. Moreover, because proteins remained in their tertiary conformation and protein complexes are not dissociated by homogenization, if the antibody recognizes part of the target protein that is in close proximity to the binding domain of a partner or hidden inside the native structure of the protein, the IP can be compromised. It is thus essential to always verify the efficiency of the IP using western blot before concluding on the absence of a binding partner. Third, co-IP can generate false-positive results either by protein binding directly to the beads, or precipitated during the procedure without being part of the complex. To identify these artifacts, an IgG control is required, as well as a reciprocal IP, as described in the method presented. It is also possible to add a "pre-cleaning" procedure between steps 2.2.2 and 2.2.3 to avoid unspecific binding of protein to the beads. To do so, incubate the lysates with 50 µg of beads for 1 h at 4 °C on a roller mixer. Remove the beads with the magnetic stand and proceed to step 2.2.3. Fourth, the heavy and light chains of IgG can be the same size as the proteins of interest or binding partner, thereby masking the signal. One solution is to dissociate the IgG chains with glycine, as described in this manuscript. It is also possible to purchase secondary antibodies that only recognize native antibodies, and therefore do not bind to the denatured light and heavy chains loaded in the membrane (see Table of material). The two methods may sometimes have to be combined. Fifth, co-IP allows for the identification of a limited number of binding partners in part because of the number of antibodies that can be probed on the membrane. It also requires the pre-identification of these binding partners either by co-IF or a literature review. Finally, co-IP allows for the identification of the proteins present within a complex, and not direct interaction between two proteins.

Results from co-IP can be analyzed in different ways. It is possible to solely identify interacting partners by re-probing the same membrane with different antibodies, as described in this manuscript. It is also possible to quantify this interaction. To do so, the amount of the protein immunoprecipitated is first quantify by probing the membrane with an antibody against this protein and signal intensity is analyzed using an imaging software, as described in this manuscript. Then, the membrane is re-probed with an antibody against the binding partner, and the signal intensity also quantified. The interactions between the two proteins can then be expressed as a ratio of the amount of the binding partner on the amount of the immunoprecipitated protein. However, to allow for comparison, the different samples have to be processed at the same time and loaded on the same membrane. Biological replicates can be proceeded and analysed similarly and statistical analysis can be performed.

In the last few years, other techniques were developed to analyze PPIs. For instance, Fluorescence Resonance Energy Transfer (FRET) allows for the identification of interacting proteins through energy transfer from one tag to another only when the proteins are close enough to interact (Bajar, Wang et al. 2016); however, because it requires proteins to be tagged, this technique cannot be used in tissues. Similarly, it is possible to identify PPIs using a protein fused with a bacterial biotin ligase BirA (Fredriksson, Van Itallie et al. 2015). This ligase will add biotin on protein that come in close proximity (i.e. interact) with the chimeric protein. While this method is innovative and unbiased, it cannot be performed in tissues. Alternatively, Proximity Ligation Assay (PLA) can be used in tissues. Similar to co-IF, this assay is based on antibody affinity. For this assay, secondary antibodies are tagged with DNA sequences that can interact when they are in close proximity, i.e. upon PPIs, and form a circular DNA molecule (Weibrecht, Leuchowius et al. 2010). This circular DNA molecule is then amplified and detected using fluorescently-labeled complementary oligonucleotides. Although this assay is elegant, it requires many validation steps, and still relies on first antibody affinity and some knowledge of potential interacting partners. Finally, an unbiased alternative to the traditional IP assay has also been developed to identify PPIs. In Rapid Immunoprecipitation-Mass Spectrometry of Endogenous protein (RIME) assays, IP samples are analyzed by mass spectrometry (IP/MS)(Malovannaya, Li et al. 2010) instead of western blot analysis. The main advantage of this high throughput method is that it provides massive information about all the endogenous interacting proteins using few materials; however, it requires access to a peptide sequencing instrument (Malovannaya, Li et al. 2010).

It is important to mention that after several preliminary tests, each step of this protocol has been optimized for the mammary gland, but can surely be used for other organs, with a few modifications. For co-IF, optimal temperature for mammary gland sectioning, suitable blocking, washing and solutions, as well as antibody concentrations were all tested. For co-IP, various lysis buffer and methods of extractions were also tested, as well as elution buffers, and have a major impact on IP success. In sum, each step of this protocol is important to obtain high-quality and reproducible results with the least possible background and the most specificity. While other methods are available, co-IF followed by co-IP remained valid and simple methods to evaluate PPIs. These two techniques can be used both in tissues and cell lines, and only require a few validation steps and controls.

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Disclosure

Nothing to declare.

Table of specific reagents/equipment

Table 1 shows recipes and reagents.

Reagents/materials/devices/chemicals	Commercial name/Formula and preparation steps	Vendors and locations	Catalogue number
Mice strain and stage	C57BL/6 Femals; pregnancy day18 (P18), and lactation day14 (L14), Charles River Canada	St. Constant, Quebec, Canada	
PBS 10X (stock)	1) Dissolve 80g NaCl (F.W.: 58.44), 2g KCl (F.W. 74.55), 26.8g Na2HPO4•7H2O (F.W. 268.07) and 2.4g KH2PO4 (F.W.:136.09) in 800ml distilled water. 2) Adjust the PH to 7.4 3) Add water to reach to the 1 litre final volume.		
TBS 10X (stock)	1) Dissolve 60.5g TRIS, 87.6g NaCl in 800ml distilled water. 2) Adjust the PH to 7.5 3) Add water to reach to the 1 litre final volume.		
Part A-Immunofluorescence			
Freezing media	VMR, frozen sections compound	VWR International, Ville Mont-Royal, QC, Canada	**95057-840**
Microtome	Microm HM525, Thermo fisher scientific HM525 NX Cryostat 115V 60Hz	Mississauga, ON, Canada	**956640**
Blades	High profile gold coated blades	C.L. Sturkey, Inc. Les Produits Scientifiques ESBE St-Laurent, QC, Canada	**BLM1001C**
Pap pen	Super PAP Pen, Thermo fisher scientific	Cedarlane, Burlington, ON, Canada	**8899**
Microscopic slides	Fisherbrand™ Superfrost™ Plus Microscope Slides	Fisher Scientific, Burlington, ON, Canada	**12-550-15**
Formaldehyde	Formadehyde	BioShop Canada Inc, Burlington, ON, Canada	**FOR201.1**
Bovine Serum Albumin (BSA)	Santa Cruz Biotechnology, Inc, California, USA		
Blocking solution	3% BSA in TBS		
Wash solution	TBS-Tween 20 0.1%		
Polysorbate 20	Tween 20, Sigma-Aldrich	Oakville, ON, Canada	**P 9416**
Mounting media	Fluoromount-G	Cedarlane, Burlington, ON	**17984-25(EM)**
First & secondary antibodies	E-Cadherin (4A2) Mouse mAb (#14472s) 1/50 (Cell Signaling) with anti-mouse IgG Fab2 Alexa Fluor 555 (#4409s), Cell Signaling Claudin-7 (#34-9100) 1/100 (Life Technologies) with anti-rabbit IgG Fab2 Alexa Fluor 488 (#4412s) (Cell Signaling) β-Catenin Antibody (C-18): sc-1496 (SANTA CRUZ) with anti-Goat IgG (H+L) Alexa Fluor 568 (#A11057), Molecular Probe (Fisher Scientific) Connexin26 (#33-5800) 1/75 (Life Technologies) with anti-mouse IgG Fab2 Alexa Fluor 647 (#4410s)	Cell Signaling, Beverly, MA, USA Life technologies, Waltham, MA, USA & Cell Signaling, Beverly, MA, USA Santa Cruz Biotechnology, Inc, California, USA; Fischer Scientific, Burlington, ON, Canada Life technologies, Waltham, MA, USA & Cell Signaling, Beverly, MA, USA	**Mentioned in Column B**
Nuclei stain	DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) 1/1000 in PBS	Fisher Scientific, Burlington, ON, Canada	**D1306**
Fluorescent microscope	Nikon A1R+ confocal microscopic laser equipped with a spectral detector	Nikon Canada, Mississauga, On, Canada	
Software of IF images analysis	NIS-elements software (version 4)	Nikon Canada, Mississauga, On, Canada	
Part B-Immunoprecipitation			
Triple-detergent Lysis buffer (100ml) pH=8.0	1) Mix 50mM TRIS (F.W.: 121.14), 150mM NaCl (F.W.: 58.44), 0.02% Sodium Azide, 0.1% SDS, 1% NONIdET P40, 0.5% Sodium Deoxycholate in 80ml distilled H2O. 2) Adjust the PH to 8.0 with HCl 6N (~0.5ml). 3) Adjust the volume to 100ml. Keep it in fridge. At the day of protein extraction, use 1/100 NaVo3, 1/100 protease/phosphatase inhibitor and 1/25 NAF in calculated amount of Triple detergent lysis buffer: Sodium Fluorid (stock) solution 1.25M (F.W. 41.98), Sodium Orthovanadate (stock) Solution 1M (F.W.: 183.9)		
Protease/phosphatase inhibitor	Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	Fisher Scientific, Burlington, ON	**78441**
Protein dosage	Pierce BCA protein assay kit	Thermo Scientific, Rockford, Illinois, USA	**23225**
Tissue grinder	Power 125, Model FTH-115	Fisher Scientific, Burlington, ON	**FTH-115**
Magnetic beads and stand	PureProteome™ Protein G Magnetic Bead System (LSKMAG02)	Millipore, Etobicoke, ON, Canada	
Wash solution for IP	PBS or PBS-Tween20 0.1% depending to the step		
Primary antibodies for immunoprecipitation	IgG Rabbit (rabbit (DA1E) mAb IgG Isotype control (#3900s) (Cell Signaling) 0.5 µl/200 µl IgG Mouse mouse (G3A1) mAb IgG Isotype control (#5415s) (Cell Signaling) 0.5 µl/200 µl Connexin43 (#C6219) (Sigma-Aldrich) 4 µl/200 µl E-cadherin (4A2) Mouse mAb (#14472s) (Cell Signaling) 1 µl/200 µl	Cell Signaling, Beverly, MA, USA Cell Signaling, Beverly, MA, USA Sigma-Aldrich, Oakville, ON, Canada Cell Signaling, Beverly, MA, USA	**Metioned in coulmn B**
Laemmli buffer	4x Laemmli Sample Buffer (Add β-mercaptoethanol following manufacturer recommendation)	BIO-RAD, Mississauga, Ontario, Canada	**1610747**
Acidic glycine	0.2 M glycine; adjust pH=2.5 with HCl	Fisher Scientific, Burlington, ON	**PB381-5**
Tris	1 M (pH=8)	Fisher Scientific, Burlington, ON	**BP152-1**
SDS-PAGE acrylamide gels	TGX Stain-Free™ FastCast™ Acrylamide Solutionss (7.8%, 10%, 12%)	BIO-RAD, Mississauga, ON, Canada	**1610180 -5**
Running buffer 10x	Tris 30.3g/glycine 144.1g /SDS 10g in 1 litre distilled water	BIO-RAD, Mississauga, ON, Canada	**1704272**
Membranes	PVDF membranes, Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit	BIO-RAD, Mississauga, ON, Canada	**1704272**
Transfer method	Trans-Blot Turbo Transfer System	BIO-RAD, Mississauga, ON, Canada	**1704155**
Dry Milk	Fat Free Instant Skim Milk Powder, Carnation	Smucker Food of Canada Co, Markham, ON, Canada	
Blocking solution for blots	5% dry milk in TBS-Tween 20 0.1%		
Washing solutions for blots	TBS-Tween 20 0.1%		
Primary and secondary antibodies for blots (10ml)	Connexin43 (#C6219) (Sigma-Aldrich) 1/2500 with HRP-conjugated Veriblot for IP secondary antibody (ab131366) 1/5000 (Abcam, Toronto, ON, Canada) E-cadherin (24E10) rabbit mAb 1/1000 (#3195s) (Cell Signaling) 1/1000 with HRP-conjugated Veriblot for IP secondary antibody (ab131366) 1/5000 (Abcam, Toronto, ON, Canada) Claudin-7 (#34-9100) (Life technologies) 1/1000 with HRP-conjugated Veriblot for IP secondary antibody (ab131366) 1/5000 (Abcam, Toronto, ON, Canada) Claudin3 (#34-1700) (Life technologies) 1/1000 with HRP-conjugated Veriblot for IP secondary antibody (ab131366) 1/5000 (Abcam, Toronto, ON, Canada)	Sigma-Aldrich, Oakville, Ontario & Abcam, Toronto, ON, Canada Cell Signaling, Beverly, MA, USA & Abcam, Toronto, ON, Canada Life technologies, Waltham, MA, USA & Abcam, Toronto, ON, Canada Life technologies, Waltham, MA, USA & Abcam, Toronto, ON, Canada	**Mentioned in column B**
Luminol solution for signal detection on blots	Clarity™ Western ECL Blotting Substrate	BIO-RAD, Mississauga, ON, Canada	**1705061**
Imaging blots	ChemiDoc MP imaging system	BIO-RAD, Mississauga, ON, Canada	**1708280**
Analayzing blots	ImageLab 5.2 software	BIO-RAD, Mississauga, ON, Canada	

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2.3 Exposure to an Environmentally Relevant Mixture of Brominated Flame Retardants Decreased p-β-Catenin^{ser675} Expression and its Interaction with E-Cadherin in the Mammary Glands of Lactating Rats

Titre en français : Une exposition à un mélange de Retardateurs de Flammes Bromés diminue l'expression de p-β-Caténine^{Ser675} et inhibe l'interaction entre p-β-Caténine^{Ser675} et E-cadhérine dans les glandes mammaires de rats

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Elham Dianati performed most of the experiments of the manuscript, contributed to experimental design and wrote the manuscript.

Mike Wade, Bernard Robaire and Barbara Hales contributed to the design of animal study, as well as revision of the manuscript.

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Abstract

Proper mammary gland development and function require precise hormonal regulation and bidirectional cross talk between cells provided by means of paracrine factors as well as intercellular junctions; exposure to environmental endocrine disruptors can disturb these processes. Exposure to one such family of chemicals, the brominated flame retardants (BFR), is ubiquitous. Here, we tested the hypothesis that BFR exposures disrupt signaling pathways and intercellular junctions that control mammary gland development. Before mating, during pregnancy and throughout lactation, female Sprague-Dawley rats were fed diets containing that BFR mixture based on house dust, delivering nominal exposures of BFR of 0 (control), 0.06, 20 or 60 mg/kg/day. Dams were euthanized and mammary glands collected on postnatal day 21. BFR exposure had no significant effects on mammary gland/body weight ratios or the levels of proteins involved in milk synthesis, epithelial-mesenchymal transition, cell-cell interactions, or hormone signalling. However, BFR exposure (0.06 mg/kg/day) down-regulated phospho-ser675 β-catenin (p-β-cat^{Ser675}) levels in the absence of any effect on total β-catenin levels. Levels of p-CREB were also down-regulated, suggesting that PKA inhibition plays a role. p-β-cat^{Ser675} co-localized with β-catenin at the mammary epithelial cell membrane, and its expression was decreased in animals from the 0.06 and 20 mg/kg/day BFR treatment groups. While β-Catenin signaling was not affected by BFR exposure, the interaction between p-β-cat^{Ser675} and E-cadherin was significantly reduced. Together, our results demonstrate that exposure to an environmentally relevant mixture of BFR during pregnancy and lactation decreases p-β-cat^{ser675} at cell adhesion sites, likely in a PKA-dependant manner, altering mammary gland signaling.

Keywords: Endocrine disruptors, Brominated flame retardants, Mammary gland, E-cadherin, p-β-catenin^{ser675}

Introduction

Brominated flame retardants (BFR) are incorporated into many consumer products to reduce the flammability of combustible materials and to meet fire safety standards (Watanabe and Sakai 2003). Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCDD) have been used widely as additive BFR in North America (Alaee, Arias et al. 2003, Stapleton, Dodder et al. 2005). Because they do not form covalent bonds within the polymer matrices to which they are added, PBDEs and HBCDDs are easily released into the environment, resulting in ubiquitous human exposure (Besis

and Samara 2012). In 2008 the Government of Canada adopted a regulation prohibiting the manufacture of certain PBDEs congeners and the use, sale, offer for sale and import of those PBDEs, as well as mixtures, polymers and resins containing these substances (Environment (Canada 2008). Regulatory action was also adopted for HBCDDs, and should come into force in 2017 (Environment Canada 2010). However, because PBDEs and HBCDDs are released from existing furnishings and other common household items and are persistent, human exposure will likely continue for many years.

The main routes of exposure of BFR are through inhalation and ingestion of dust, as well as dietary consumption (Besis and Samara 2012). BFR are detected in breast milk, adipose tissue, serum and hair and are bioaccumulative (Hites 2004, Toms, Hearn et al. 2009, Zota, Linderholm et al. 2013). Various BFR congeners have been reported to act as endocrine disruptors. Exposure to PBDEs has been associated with adverse effects on male and female reproductive organs and on steroid and thyroid hormone signalling (Lilienthal, Hack et al. 2006, Chevrier, Harley et al. 2010, Ernest, Wade et al. 2012, Lefevre, Berger et al. 2016, Lefevre, Wade et al. 2016). A previous study showed a significant delay in ductal outgrowth in the mammary gland at PND21 after perinatal exposure to DE-71 (Kodavanti, Coburn et al. 2010). *In vitro*, decreased apoptosis and increased proliferation were observed when MCF-7 breast cancer cells were exposed to a combination of PBDEs (BDEs 47, 99, 100 and 209) and estradiol (Kwiecinska, Wrobel et al. 2011).

Mammary gland development and function involve bidirectional communication, composed of paracrine factors and cell junctions between luminal and myoepithelial cells, and are driven by multiple systemic hormones (Gudjonsson, Adriance et al. 2005, Hennighausen and Robinson 2005, Stewart, Simek et al. 2015). Among others, estrogens (E2), progesterone, prolactin, Jak2/Stat5 signaling pathways and ERBB2/HER2/NEU receptor tyrosine kinases are involved in tertiary ductal branching, alveologenesis and the synthesis of milk during pregnancy and lactation (Humphreys, Lydon et al. 1997, Gallego, Binart et al. 2001, Hewitt, Bocchinfuso et al. 2002, Jackson-Fisher, Bellinger et al. 2004). Thus, changes in hormonal balance can perturb mammary gland development and function. In accordance, exposure to endocrine disruptors at sensitive periods of life, such as the perinatal life, puberty or pregnancy, have been linked to developmental defect and breast cancer (Fenton 2006). Those sensitive periods involved proliferation, differentiation and apoptosis events that are tightly orchestrated by signaling pathways.

Intercellular junctions are also required for normal mammary gland function. Dysregulation of junctional proteins is associated with breast developmental defects and cancer (Hatsell, Rowlands et al. 2003, Osanai, Murata et al. 2007, Plante and Laird 2008, Lanigan, McKiernan et al. 2009, Stewart, Simek et al. 2015). Down-regulation and altered localization of junctional proteins are observed following exposure to a variety of pollutants, suggesting that environmental chemicals may alter mammary gland function (Plante, Charbonneau et al. 2002, Salian, Doshi et al. 2009, Leithe, Kjenseth et al. 2010, Trosko 2011). We recently showed that the composition of gap, adherens and tight junctions in junctional nexuses and the expression of their components vary during mammary gland development (Dianati, Poiraud et al. 2016). Notably, gap junction proteins, Cx26, Cx30 and Cx32, adherens junction proteins, E-cadherin and β-catenin, as well as tight junction proteins, claudin 3 and claudin 7, were all up-regulated, while Cx43 and P-cadherin were down-regulated during pregnancy and/or lactation (Dianati, Poiraud et al. 2016). These data suggest stage-dependent requirements in cell-cell interactions during mammary gland development and a role for hormones and paracrine factors in their regulation (Risek, Klier et al. 1995, Nguyen, Parlow et al. 2001, Rubenstein, Guan et al. 2003). Junctional proteins and their binding partners are also involved in regulating cellular signaling and transduction of signals. Among others, β-catenin plays a pivotal role in mammary gland development and function (Hatsell, Rowlands et al. 2003, Carraway, Ramsauer et al. 2005). β-Catenin can either be bound to E-cadherin at the junctional nexus or act as a transcription factor in collaboration with TCF/Lef DNA binding proteins. This dual role is controlled, in part, by phosphorylation of β-catenin by various kinases, including PKA, CKια, Akt and GSK3β (Roura, Miravet et al. 1999, Liu, Li et al. 2002, Taurin, Sandbo et al. 2006, Spirli, Locatelli et al. 2013). Thus, dysregulation of β-catenin has been associated with abnormal mammary gland development, with the epithelial-to-mesenchymal transition (EMT) and with breast cancer (Hatsell, Rowlands et al. 2003, Carraway, Ramsauer et al. 2005).

In the current study we tested the hypothesis that exposure to common household flame retardants during pregnancy and lactation alters mammary gland function and signaling pathways. Specifically, we evaluated the effects of an environmentally-relevant mixture of PBDE and HBCDD congeners on mammary gland function and development, focusing on: 1) the milk synthesis pathway, 2) hormonal receptors, and 3) the expression, localization and interactions of junctional proteins.

Material and methods

Exposure to a mixture of BFRs

Formulation of the mixture of BFR and preparation of the diets were described previously (Berger, Lefevre et al. 2014, Lefevre, Berger et al. 2016, Lefevre, Wade et al. 2016, Tung, Yan et al. 2016, Tung, Kawata et al. 2017). Briefly, the BFR dietary mixture contained three different technical PBDE flame retardant mixtures (DE-71, DE-79 and BDE-209) and an HBCDD mixture that were combined to yield proportions of PBDE and HBCDD congeners that were comparable to median levels measured in Boston house dust (Allen, McClean et al. 2008, Stapleton, Allen et al. 2008). Sufficient mixture was combined with powdered diet (0, 0.75, 250 or 750 mg of BFR mixture/kg diet) to deliver a daily nominal dose of 0, 0.06, 20 or 60 mg/kg of body weight/day (mg/kg/day), respectively. Our lowest dose approximated the maximum human exposure, which was calculated based on the dust ingestion rate of 100 mg/day in children and then converted to rat by scaling ratio of 1:6.9 (human to rat body surface area) (Allen, McClean et al. 2008, Stapleton, Allen et al. 2008). Powdered diets were mixed thoroughly, pelleted and stored at 4 C for no more than 1 month prior to feeding.

Animals

Animals and treatment methods were previously described (Tung, Yan et al. 2016). Briefly, virgin female Sprague-Dawley rats were obtained from Charles River Laboratories (Charles River, St-Constant, QC, Canada). Animals were housed individually in accordance with the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council Protocol 2012-015. Food and water were provided ad libitum; the rats were housed in rooms maintained with a 12L: 12D photoperiod, 40-70% humidity at 20-24°C. Exposure began at least one week prior to mating and was maintained during gestation and lactation. Female rats were acclimated to control diets for one week and then randomly assigned to one of the four diet groups prior to the mating. Females in proestrus, as assessed by vaginal cytology, were then caged with proven breeder male Sprague-Dawley rats (maintained on the control diet) overnight. A sperm-positive vaginal swab on the following morning indicated mating and this was considered as gestational day 0. Females were returned to their home cage and provided with water and the appropriate dietary mixture *ad libitum* throughout gestation and lactation. All females were allowed to deliver (denoted as PND 0), dams were euthanized within 15 minutes after being separated from pups at PND 21 by abdominal aorta exsanguination under isoflurane anaesthesia. Litter size was balanced at 8 pups per dam at postnatal day PND4. At the time of necropsy, the mammary glands were

sampled and preserved differently depending on the downstream applications. Abdominal mammary glands (pair 4) were weighed and immediately flash-frozen in liquid nitrogen; inguinal mammary glands (pair 5) were also flash-frozen. These glands were used for western blot and immunoprecipitation analyses. Mammary gland pairs 2-3 (upper-thoracic and thoracic) were embedded in Tissue-Tek OCT cryomounting compound (VWR International, Ville Mont-Royal, QC, Canada) on dry ice or fixed in 4% formalin and used for histology and immunofluorescence. All unfixed samples were stored at -80°C.

Western blot

Frozen tissues were homogenized in ice-cold triple detergent lysis buffer (pH 8) (Tris 50 mM, NaCl 150 mM, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate) supplemented with 1.25 M of NaF, 1 M of NaVO3 and Halt Protease and Phosphatase Cocktail Inhibitor (Fisher Scientific) and centrifuged at ≥4000 rpm (10 minutes at 4°C). Aliquots of the supernatant were stored at -80 °C. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, Illinois, USA). To perform semi-quantitative western blot analysis (Taylor et al., 2013), total proteins were loaded onto TGX Stain-Free™ Acrylamide gels (BIO-RAD, Mississauga, Ontario, Canada). Immediately after electrophoresis, the gels were transferred onto PVDF membranes using the Trans-Blot Turbo Transfer System (BIO-RAD). Total lane proteins were visualized using the ChemiDoc MP imaging system (BIO-RAD) and quantified using ImageLab 5.2 software (BIO-RAD) for normalization. Membranes were blocked with 5% dry milk in TBS-Tween 0.1% and probed overnight at 4°C with primary antibodies diluted in 5% dry milk or 3% BSA in TBS-Tween 0.1% (Supplementary Table 1). Membranes were washed 3 times, for 5 minutes each, with TBS-Tween 0.1% and probed with secondary antibodies in TBS-Tween 0.1% (Supplementary Table 1). The signal was obtained using Clarity™ Western ECL Blotting Substrate (BIO-RAD) and visualized using the ChemiDoc MP imaging system (BIO-RAD). The density of each band was normalized to the total proteins in the lane using ImageLab 5.2 software (BIO-RAD). Protein extracts from 9 randomly picked animals per group were analysed (N=9).

The acquisition of microscopic images and analysis of immunofluorescence

Immunofluorescence staining and analyses were performed as previously described (Dianati and Plante, 2017). Briefly, tissue cryosections (7 µm) were fixed in 4% formaldehyde and blocked in 3% BSA dissolved in TBS-Tween 0.1%. Primary antibodies were diluted in TBS-Tween 0.1% solutions and sections were incubated for 60 minutes at room temperature (or overnight at 4°C) with primary

antibodies (Supplementary Table 2). Staining was followed by three washes (5 minutes each) with TBS-Tween 0.1%. Sections were incubated with the appropriate secondary antibodies (Supplementary Table 2). Staining was followed by another series of three washes. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI), and slides were mounted with Fluoromount-G (Cedarlane, Burlington, ON, Canada). Immunofluorescence images were obtained with a Nikon A1R+ confocal microscopic laser equipped with a spectral detector and analyzed using NIS-elements software (version 4). P-β-catser675 signal was quantified using the area of the immunofluorescence staining relative to that of the nucleus (DAPI) staining for each image. Cryosections from 4 randomly picked animals per group were analysed (N=4); for each animal, 4 random areas of the section were quantified.

Immunoprecipitation

Immunoprecipitation analyses were done as previously described (Dianati and Plante, 2017). Briefly, the lysates used for immunoprecipitation were prepared from flash-frozen tissue, as described in the western blot section. Immunoprecipitation was performed using the PureProteome™ Protein G Magnetic Bead System (LSKMAGG02) (Millipore, Etobicoke, ON, Canada); 200 µl of lysates containing a total of 500 µg protein were incubated overnight at 4°C with slow agitation with the primary antibody (Supplementary Table 3). The next day, 50 µl of magnetic beads were added to the incubated lysate-antibody solution, and the bead-antigen-antibody complex was incubated at room temperature for 90 minutes with slow agitation. After washes, the bead-antibody-protein complexes were dissociated using two consecutive incubations with 20 µl of glycine 0.2M (pH 2.5) to remove the IgG. Immunoprecipitation was followed by western blot analysis (Supplementary Table 3), as described above, using immun-Blot® low fluorescence PVDF membranes (BIO-RAD). Membrane signals were detected using either Clarity™ Western ECL Blotting Substrate (BIO-RAD) for HRP antibodies, or directly observed using the ChemiDoc MP imaging system for fluorophores (BIO-RAD). Protein-protein interaction was quantified as a ratio of the signal of the interacting protein to the signal of the precipitated target using ImageLab 5.2 software (BIO-RAD). Mammary gland protein extract from 4 randomly picked animals per group were analysed (N=4).

Masson's trichrome staining

Cryosections of mammary glands (N=4) were cut (7 µm) and kept at -80°C. At the time of coloration, sections were retrieved from -80°C and fixed in Bouin's overnight. Sections were then sequentially stained with Weigert's iron hematoxylin (10 minutes), Biebrich scarlet-acid fuchsin (10

minutes), phosphomolybdic-phosphotungstic acid (20 minutes) and aniline blue (5 minutes). Sections were washed with warm tap water for 10 minutes between each coloration step. Finally, the sections were treated with 1% acetic acid for 5 minutes, dehydrated in an alcohol series (95% and 100%), cleared in xylene for 5 minutes and mounted using Permount (Fisher Scientific, Burlington, ON). Cryosections from 4 randomly picked animals per group were analysed (N=4); for each animal, ≥4 random areas of the section were observed.

Statistical analyses

All statistical analyses were done using GraphPad Prism, version 6.01. Normal distribution of each data set and the presence of outliers were verified with a D'Agostino & Pearson omnibus normality test and Grubbs' test (ESD method), respectively. For the data with normal distribution (alpha=0.05), P-value was calculated using a parametric one-way ANOVA followed by Dunnett's multiple comparison tests; otherwise, a non-parametric Kruskal-wallis followed by Dunn's multiple comparison tests was used.

Results

BFRs treatments did not affect markers of milk synthesis or mammary gland function

Effects of the mixture of BFR on pregnancy outcomes, pup growth and development, as well as on pups' mammary gland development, are reported elsewhere (Tung, Yan et al. 2016, Tung, Kawata et al. 2017) or currently under investigation. Exposure to the BFR mixture had no effects on dams mammary gland or body weights (data not shown), or on the ratio of mammary gland to the body weight of the dams (supplementary Figure 1A). Mammary gland structure also remained unchanged between control and BFRs treated animals (Supplementary Figure 1B). However, T4 serum levels were reduced in dams, suggesting potential effects on mammary gland signaling or protein expression (Tung et al., 2016). The mammary gland levels of β-casein, a major milk protein, (Supplementary Figure 1C), or of Stat5 and p-Stat5^{tyr694}, the down-stream effector of the prolactin receptor/Jak2/Stat5 signaling pathway, were not affected in response to BFR treatments (Supplementary Figure 1D and 1E). We also evaluated the effects of BFR on indicators of EGF, estrogen and thyroid hormone signalling pathways. No significant changes were observed in the levels of EGFR/ErbB-1, pEGFR (Tyr1068), ErbB2, ERα or thyroid hormone receptors α or β in the treated groups compared to controls (Supplementary Figure 2).

Effects of BRF exposure on β-catenin levels and phosphorylation

We next investigated whether the dual roles of β-catenin as an adherens junction protein and/or in signal transduction were affected by treatment with BFR. Western blot analysis showed that the total protein levels of β-catenin remained unchanged after exposure to BFR (Figure 1A). Similarly, the levels of non-p-β-cat^{Ser45} and p-β-cat^{Tyr654} were not altered in the mammary glands from BFR treatment groups (Figure 1B, 1D). Interestingly, we observed a significant down-regulation of approximatively 50% in p-β-cat^{Ser675} levels in animal treated with the low BFR dose (0.06 mg/kg/day) compared to control (Figure 1C). To investigate whether p-β-cat^{Ser675} down-regulation was associated with a decrease in the levels or change in the activity of Akt (protein kinase B) and glycogen synthase kinase 3beta (GSK-3β), kinases known to be involved in its early phosphorylation on serine (Ser) 552 and Ser33/Ser37/Thr41, respectively, we determined their total protein levels as well as the levels of phosphorylated forms by western blotting. Protein levels of Akt, p-Akt^{Ser473}, GSK-3β and p-GSK-3β^{Ser9} were not altered by BFR exposure, suggesting that the activity of these kinases was not altered (Figure 2A-D). To evaluate PKA activity, we quantified the levels of CREB and phospho-CREB, a direct target of PKA (Rosenberg et al., 2002). While protein levels of PKA and CREB were unchanged, a significant decrease (~40%) in p-CREB was observed in groups in the 0.06 and 20 mg/kg/day BFR treatment groups (Figure 2F, 2G), suggesting a decrease in PKA activity.

β-Catenin signaling is not affected by BFR exposure

Previous studies have shown that phosphorylation of β-catenin at Ser675 increases its binding to transcriptional co-activators and promotes TCF/LEF-dependant gene transcription (Taurin, Sandbo et al. 2006). We examined the interaction of p-β-cat^{Ser675} with Lef1 and expression of their downstream targets to investigate whether p-β-cat^{Ser675} down-regulation is associated with a change in the β-catenin/Lef1 signaling pathway (Figure 3). Surprisingly, immunoprecipitation analysis revealed no interaction between p-β-cat^{Ser675} and Lef1 in control or treated animals (Figure 3A). Consistently, analysis of TCF/LEF-dependant gene expression showed that BFR exposure did not alter the expression of TCF1, c-Jun, c-Myc or Met in the rat mammary gland (Figure 3 B-E).

Endogenous p-β-Cat^{Ser675} is localized at the cell membrane

Because our results did not show alterations in the expression of known β-catenin downstream targets (Figure 3 B-E), we compared the localisation of β-catenin and p-β-cat^{Ser675} in mammary glands from control and BRF exposed dams using confocal immunofluorescence. In control animals, endogenous p-β-cat^{Ser675} resides at basolateral and apical areas of the membrane where it co-localizes

with β-catenin (Figure 4A). Interestingly, in animals exposed to the 0.06 and 20 mg/kg/day BFR mixtures, a lower expression of p-β-cat^{Ser675} was observed (Figure 4B, 4C and 4E). In animals from the highest dose treatment groups, a non-significant decrease was observed (Figure 4D and 4E).

Expression of adherens, tight and gap junctions proteins and of markers of the epithelial-mesenchymal transition (EMT) were not altered by exposure to BFR

Since the localization of p-β-cat^{Ser675} at the cell membrane of epithelial cells was decreased in animals from the 0.06 and 20 mg/kg/day BFR treatment groups, we investigated whether other junctional proteins or markers of EMT were affected by the treatment. The expression levels of vimentin and N-cadherin, markers of mesenchymal cells, (Supplementary Figure 3A and 3B), of E- and P-cadherin, adherens junction proteins expressed in luminal and myoepithelial cells, respectively (Supplementary Figure 3C and 3D), of claudin-7 and occludin, components of tight junctions (Supplementary Figure 3E and 3F), and of connexin 43 (Cx43) and connexin 32 (Cx32), constituents of gap junctions (Supplementary Figure 3G and 3H), were not different between control and BFR-exposed rats. The localisations of E-cadherin, vimentin, Cx43 and Cx32 were also not affected (Supplementary Figure 4).

Effects of exposure to BFR on the interaction of p-β-cat^{ser675} with E-cadherin

We investigated the interaction of p-β-cat^{Ser675} with E-cadherin, the marker for adherens junctions, since the localization of p-β-cat^{Ser675} at the cell membrane of epithelial cells was decreased in animals from the 0.06 and 20 mg/kg/day BFR treatment groups. Co-immunoprecipitation analysis showed that while E-cadherin was equally precipitated in control and treated animals (Figure 5A top panel and 5B), the interaction of β-catenin with E-cadherin tended to be lower (P = 0.07) in the mammary glands of animals from the 0.06 mg/kg/day treatment group (Figure 5C). Interestingly, our results demonstrated that in this treatment group, the p-β-cat^{Ser675}/E-cadherin interaction was significantly decreased (Figure 5D); a non-statistically significant suppression was also observed in animals from the 20 and 60 mg/kg/day BFR treatment groups (Figure 5D). Interaction between p-β-cat^{Ser675} and E-cadherin was confirmed by performing the reciprocal co-IP (Supplementary Figure 5).

Discussion

Because mammary gland development requires precise hormonal regulation and environmental endocrine disruptors may disturb this process, here, we investigated the effect(s) of an exposure to an

environmentally relevant BFR mixture of during pregnancy and lactation on mammary gland development and function. Interestingly, we found that p-β-cat^{Ser675} levels were decreased in dams from the low dose, environmentally relevant, BFR treatment group. Furthermore, p-β-cat^{Ser675} was localized to the cell membrane in the lactating gland. An inhibition of the interaction of p-β-cat^{Ser675} with E-cadherin was observed that is likely linked with an effect on PKA activity as levels p-CREB, a direct target of PKA, were also down-regulated.

While several studies have examined the effects of in utero and early life exposures to PBDEs and HBCDD on a range of aspects of growth and development, only one determined the effects on mammary gland development in female offspring (Kodavanti et al., 2010); in this study, exposure was to a technical mixture, DE-71. To the best of our knowledge, our research is the first to evaluate the effects of BFR exposure during gestation and lactation on mammary gland function and development in dams. Previously, we reported that exposure to the environmentally relevant mixture of BFR found in house dust in utero and during lactation decreased T4 levels, but had no impact on the weights of offspring at the time of weaning, although at later ages body weights were significantly reduced in the two highest dose treatment groups (Tung et al., 2016). This observation would suggest that exposure to BFR does not affect the lactation capacity of the dams, but could still dysregulate signaling pathways. We did not have observe treatment-related changes in tissue structure and the protein levels of a broad array of markers of milk synthesis (β-casein) or in measures of hormonal responsiveness (total or phosphorylated Stat5, ER alpha or thyroid hormone receptors), epidermal growth factor signalling (EGF receptors ErbB-1 and 2, phosphor-ErbB1), adhesion proteins (N-, P- or E-cadherin), intermediate filaments (vimentin), tight junctions (claudin-7, occludin) or gap junctions (Cx32 and Cx43). However, β-catenin activation (p-β-cat^{Ser675}) and interaction with E-cadherin were perturbed by low dose treatment with BFR.

p-β-cat^{Ser675} expression and interaction with E-cadherin were perturbed by exposure to BFR

Exposure to the BFR mixture caused a reduction in the levels of p-β-cat^{Ser675} immunoreactivity in the dam mammary gland. Notably, exposures to the lowest dose of BFR had the greatest effect, with a reduction in mean levels to roughly 50% of control, measured using two different assays. In dams receiving the intermediate dose, the decrease in p-β-cat^{Ser675} was significant only when immunofluorescence images were quantified. In dams exposed to the highest dose, p-β-cat^{Ser675} levels were not different from those observed in control samples. β-catenin phosphorylation at other

residues was not altered by BFR exposure. Similarly, the levels of p-β-cat^{Ser675} associated with immunoprecipitated E-cadherin were more dramatically reduced in mammary glands from dams from the lowest dose treatment groups, whereas samples from dams receiving the higher doses did not differ from control.

It has been suggested that exposure to endocrine disruptors in sensitive periods of life can promote breast cancer (Fenton, 2006; Cohn et al., 2012). Interestingly, higher levels of polychlorinated biphenyls (PBCs) in early postpartum serum samples in women were correlated with a six fold increased risk of breast cancer in women (Cohn et al., 2012). In our study, we found a significant decrease in β-catenin phosphorylation at Ser675 and a reduced association with E-cadherin but we did not observe a change in β-catenin levels or localisation. Loss of cadherin-bound β-catenin is correlated significantly with poor clinical outcome in breast cancer (Dolled-Filhart et al., 2006); perturbing the role of β-catenin in signal transduction leads to precocious alveologenesis, adenocarcinoma, hyperplasia, hyperbranching and lactation defects (Incassati et al., 2010; Tepera et al., 2003; Teuliere et al., 2005). Interestingly, exposure to the lowest doses of BFR significantly downregulated the p-β-cat^{Ser675} levels in dams in the absence of effects on overall β-catenin levels. The consequences of reduced p-β-cat^{ser675} for the function and long term health of the lactating mammary gland is not clear. Thus, whether or not these modulations are associated with increased risk of breast cancer remains to be evaluated.

β-Catenin possesses many putative phosphorylation sites. Phosphorylation at Ser33, Ser37 and Thr41 by GSK3-β, and at Ser45 by the priming kinase CKια, increases ubiquitination and degradation by proteasomes (Liu et al., 2002). Alternatively, phosphorylation at Tyr654 increases the binding of β-catenin to E-cadherin in adherens junctions (Roura et al., 1999). Phosphorylation of β-catenin at Ser675 is less well studied. However, it has been demonstrated that Ser675 phosphorylation prevents β-catenin degradation and thereby promotes its transcriptional activity (Spirli et al., 2013; Taurin et al., 2006).

Consequently, we investigated the protein levels of PKA, Akt and GSK-3β, kinases that are linked to β-catenin phosphorylation (Fukumoto et al., 2001; Gordon and Nusse, 2006; Spirli et al., 2013; Taurin et al., 2006). Phosphorylation of Akt and GSK3β in BFR treatment groups did not differ from control; although phosphorylation of these kinases is commonly considered as evidence of their activation status, more studies are required to determine whether the activities of those kinases are truly unchanged. Since PKA levels are not indicative of its activation we examined the phosphorylation of

CREB, a direct target of PKA (Rosenberg et al., 2002). Levels of p-CREB were reduced in animals from the 0.06 and 20 mg/kg/day treatment groups, suggesting that treatment with BFR indeed led to an inactivation of PKA that may explain the decrease in p-β-cat^{Ser675} levels that we observed. Interestingly, alterations of the CREB family of transcription factors are observed in hormono-dependent organs, and their dysregulation are observed in tumors (Rosenberg, et al., 2002).

Since β-catenin transcriptional activation is associated with phosphorylation at Ser675 and transcriptional activity of β-catenin requires its nuclear localisation (Estus et al., 2016; Spirli et al., 2013; Taurin et al., 2006), we examined whether there was a change in the localisation of this protein after treatment with BFR. We observed a treatment-related reduction in p-β-cat^{Ser675} immunoreactivity but did not see any apparent decrease in the nuclear localization of p-β-cat^{Ser675} in mammary gland epithelial cells. Furthermore, our results showed no interaction between p-β-cat^{Ser675}/Lef1, and no changes in the expression of recognized downstream targets (Boon et al., 2002; Mann et al., 1999; Shtutman et al., 1999), suggesting that β-catenin signaling is not affected by exposure to BFR.

To further confirm the localisation and involvement of p-β-cat^{Ser675} at cell membrane, we did an immunoprecipitation assay with E-cadherin, the adherens junction component that interacts with β-catenin at the membrane of luminal epithelial cells. Our co-immunoprecipitation assays demonstrated an interaction between E-cadherin and p-β-cat^{Ser675}, thus confirming that β-cat^{Ser675} was indeed located at cell membrane. Moreover, a significant decrease in E-cadherin/p-β-cat^{Ser675} interactions was observed specifically in the mammary glands from dams in the lowest dose BFR treatment group. It is interesting that changes in p-β-cat^{Ser675} expression in the mammary glands of BFR exposed dams were observed most consistently in our lowest dose treatment groups. Non-monotonic dose response curves (NMDRs) and low-dose effects have been reported previously for endocrine active chemicals (Vandenberg et al., 2012).

Conclusion

The exposure of female rats during pregnancy and lactation to a mixture of BFR representative of those found in house dust significantly reduces the level of p-β-cat^{Ser675} expression in the mammary glands. This decrease is likely to be PKA-dependent manner and is accompanied by a decrease in p-β-cat^{Ser675}/E-cadherin interaction at the cell membrane. While there were no apparent adverse effects on the structure or function of the mammary gland during pregnancy and lactation, these effects may have significant consequences on signal transduction linked to PKA activity. Since the role of

endogenous p-β-cat^{Ser675} is not yet fully understood, further investigations should establish its role in normal mammary gland development and the consequences of its dysregulation on involution and in long-term on breast cancer.

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Supplementary Data Description

Supplementary data present histology of mammary glands and different markers which levels were not affected by the BFR treatment. These data include dam mammary gland/body weight ratios, tissue structure and levels, or localization of proteins involved in lactation, of hormonal receptors, of markers of the epithelial-mesenchymal transition and of junctional proteins. Also included in the supplementary material are the analysis of the Immunofluorescence intensity of p-β-cat^{ser675} in dam mammary glands, to complement figures 1 and 4, and the reciprocal IP to confirm the interaction between p-β-cat^{ser675} and E-cadherin.

CONFLICTS OF INTEREST

None to declare

Figure legends

Figure 1) Effects of BFR treatments on the levels of β-catenin. Total proteins were extracted from the mammary glands of control dams or dams fed a BFR mixture diet formulated to deliver a daily nominal BFR mixture dose of 0.06, 20 or 60 mg/kg of body weight/day (mg/kg/day). Quantitative

western blotting was done to evaluate β-catenin **(A)**, non-p-β-cat^{ser45} **(B)**, p-β-cat^{ser675} **(C)** and p-β-cat^{Tyr654} **(D)** protein expression. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with an ANOVA (A) or a Kruskal-wallis (B-D) statistical tests.* = p ≤0.05

Figure 2) Effects of BFR treatments on the levels of Akt, GSK3β, PKA, CREB1 and their phosphorylated forms. Total proteins were extracted from the mammary glands of control dams or dams fed a BFR mixture diet formulated to deliver a daily nominal BFR mixture dose of 0.06, 20 or 60 mg/kg of body weight/day (mg/kg/day). Quantitative western blots were done to evaluate the expression of protein kinases involved in β-catenin phosphorylation Akt **(A)**, Phospho-Akt (Ser473) **(B),** GSK-3β **(C),** Phospho-GSK-3β (Ser9) **(D)** and PKA **(E)**, and of CREB **(F)** and phospho-CREB **(G)**, a direct target of PKA. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with a Kruskal-wallis (A, C-G) or an ANOVA (B) statistical tests. * = p ≤0.05

Figure 3) Evaluation of β-catenin/Lef1 interaction and of the levels of their downstream target genes. A) p-β-cat^{ser675} was immunoprecipitated using total lysates from mammary glands from control and BFR exposed animals. Biological replicates (N = 4) were processed at the same time but loaded and transferred on different gels/blots when required. Membranes were probed with p-β-cat^{ser675}/Alexa rabbit 488 and LEF1/Veriblot HRP consecutively. **B-E)** Total proteins were extracted from the mammary glands from control, low, medium, high dose treated dams. Quantitative western blots were done to evaluate downstream targets of β-catenin-Lef1; TCF1 **(B)**, c-Jun **(C)**, c-Myc **(D)** and Met **(E)**. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with a Kruskal-wallis statistical test.

Figure 4) Localisation of endogenous β-catenin and p-β-cat^{ser675} in mammary glands from the dams. Cryosections were cut (7 µm) and processed for immunofluorescence staining. **(A-D)** Nuclei were stained with DAPI (blue). β-catenin (red) and p-β-cat^{ser675} (green) co-localized at the membrane, and the levels of p-β-cat^{ser675} differed dramatically in control and BFR-exposed animals. Images were obtained with a Nikon A1R+ equipped with a spectral detector and analyzed using NIS-elements software. (**E**) The columns represent the means ± SEM (N=4-7) for p-β-cat^{ser675} staining normalized to nuclei staining. P-values were calculated with a Kruskal-wallis statistical test. * = p ≤0.05

Figure 5) Analysis of the interactions between β-catenin and E-cadherin and between p-β-cat^{ser675} and E-cadherin. A) E-cadherin was immunoprecipitated using total lysates from mammary glands from control dams and dams fed a BFR mixture delivering nominal doses of 0.06, 20 and 60 mg/kg/day. Biological replicates (N=4) were processed at the same time but loaded and transferred on different gels/blots when required. Membranes were probed with E-cadherin/Alexa mouse 647, p-β-cat^{ser675} **/**veriblot HRP and β-catenin/Alexa mouse 488 consecutively. **B)** The protein density of precipitated E-cadherin. **C)** β-catenin/E-cadherin protein interactions calculated as the ratio of β-catenin to E-cadherin. **D)** p-β-cat^{ser675} /E-cadherin protein interactions calculated as a ratio of p-β-cat^{ser675} to E-cadherin. The columns represent the means ± SEM; P-values with a Kruskal-wallis statistical test. * = p ≤0.05

Supplementary Figure 1) Effects of exposure to BFR mixtures on dam mammary gland/body weight ratios and on proteins involved in lactation. Abdominal mammary glands (pair 4) from dams from the control and BFR mixture treatment groups (dietary administration to deliver nominal doses of 0.06, 20 and 60 mg/kg/day) were excised and weighed immediately. **(A)** The columns represent means ± SEM (N≥9) of each mammary gland normalized to the body weight. Each point represents data for one animal. **(B)** Cryosections were cut (7 µm) and processed for Masson's trichrome staining (N=4). Images were obtained with a Nikon A1R+ bright field microscopy. **(C-E)** Total proteins were extracted from the mammary glands of control dams or dams fed a BFR mixture. Quantitative western blots were done to evaluate β-casein **(C)**, Stat5 **(D)** and pStat5 **(E)** protein expression. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with a Kruskal-wallis (A, C, E) or an ANOVA (B) statistical tests.

Supplementary Figure 2) Effects of BFR exposure on the levels of EGFR/ErbB-1, pEGFR/pErbB-1, ErbB-2, ERα, PR-B, TRα and TRβ. Total proteins were extracted from the mammary glands of control dams or dams fed a BFR mixture delivering nominal doses of 0.06, 20 and 60 mg/kg/day. Quantitative western blots were done to evaluate EGFR/ErbB-1 **(A)**, pEGFR/pErbB-1 **(B)**, ErbB-2 **(C)**, ERα **(D),** PR-B **(E),** TRα **(F)** and TRβ **(G)** protein expression. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with a Kruskal-wallis (A, B, E-G) or an ANOVA (C, D) statistical test.

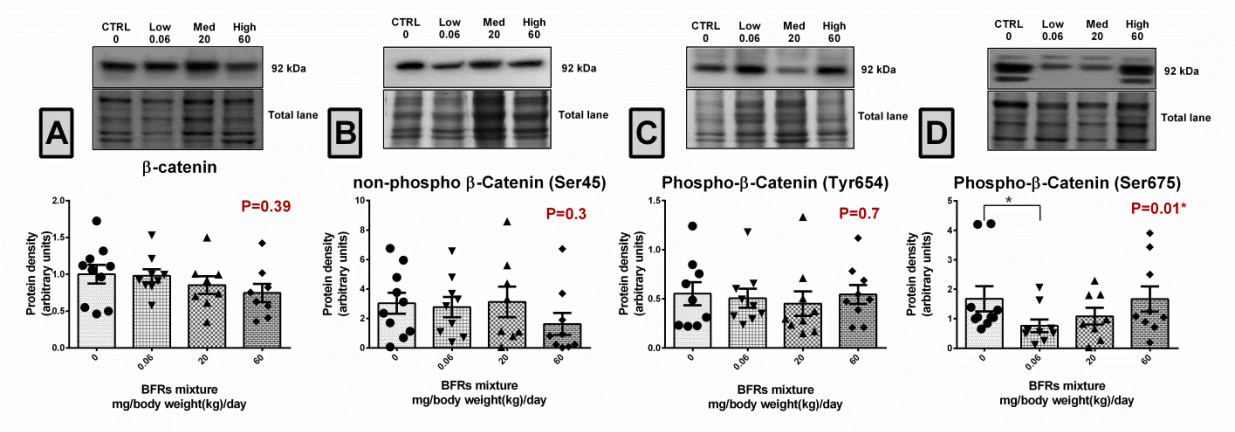
Supplementary Figure 3) Effects of dietary BFR treatments on the levels of markers of epithelial-mesenchymal transition and of junctional proteins. Total proteins were extracted from the mammary

glands from control dams and dams fed a BFR mixture delivering nominal doses of 0.06, 20 and 60 mg/kg/day. Quantitative western blots were done to evaluate Vimentin **(A)**, N-Cadherin **(B)**, P-Cadherin **(C)**, E-cadherin **(D)**, Claudin-7 **(E)**, Occludin **(F)**, Cx43 **(G)** and Cx32 **(H)** protein expression. The columns represent the means ± SEM (N=9) for each band normalized to the total protein level. P-values were calculated with a Kruskal-wallis (A, H) or an ANOVA (B-D, F, G) statistical test.

Supplementary Figure 4) Localization of E-cadherin, vimentin, Cx43 and Cx32 in dam mammary glands. Cryosections were cut (7 µm) and processed for immunofluorescence staining (N=4). **(A)** E-cadherin (red) and **(B)** Vimentin (green) **C)** Cx43 (green) and E-cadherin (red) and **(B)** Cx32 (green) localization were studied. Nuclei were stained with DAPI. Images were obtained with a Nikon A1R+ equipped with a spectral detector and analyzed using NIS-elements software.

Supplementary figure 5) Reciprocal co-IP between p-β-cat^{ser675} and E-cadherin. p-β-cat^{ser675} was immunoprecipitated (N=2) using pool of total lysates from mammary glands from control and BFR exposed animals. Membranes were probed with p-β-cat^{ser675} /HRP rabbit veriblot, E-cadherin/ HRP mouse and β-catenin/Alexa flour 488 consecutively.

Figure 1)



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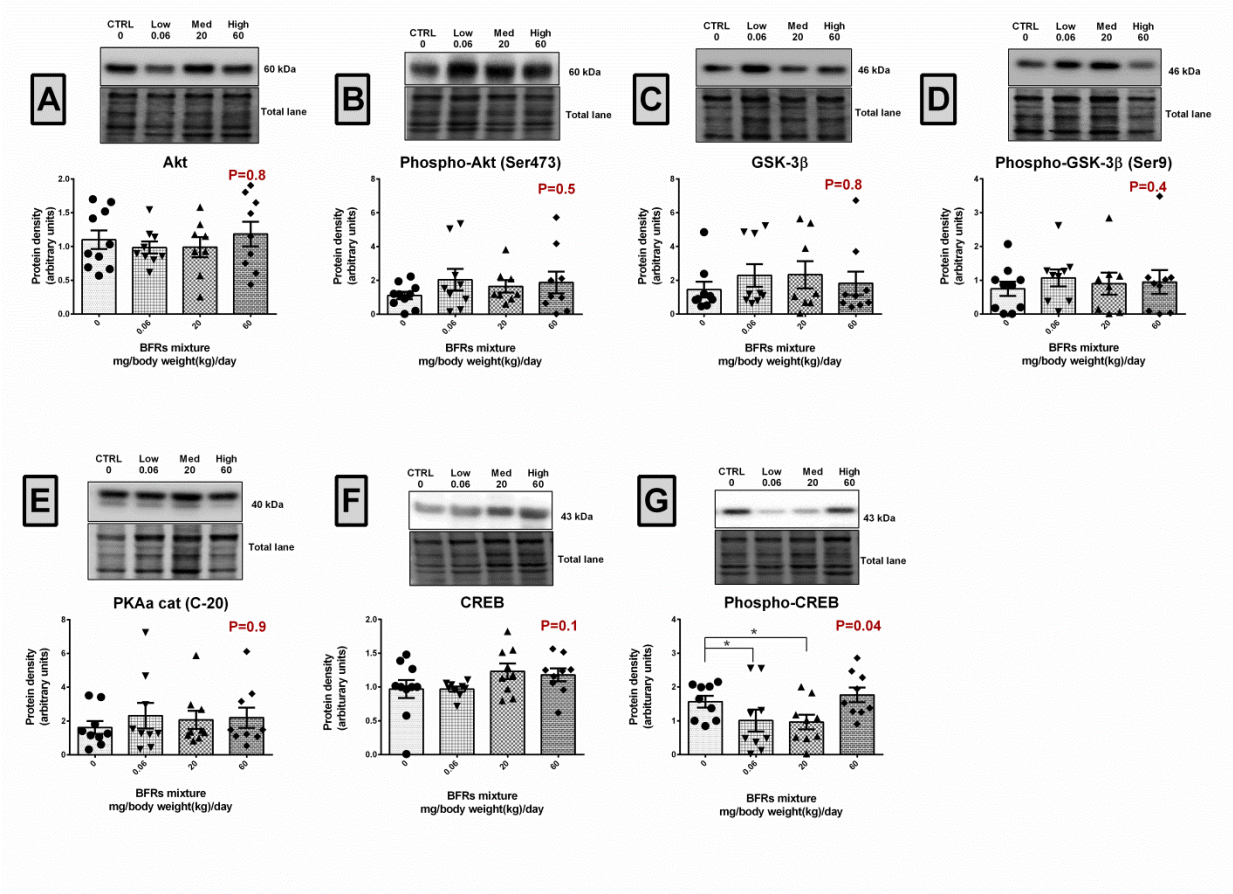
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Figure 3)

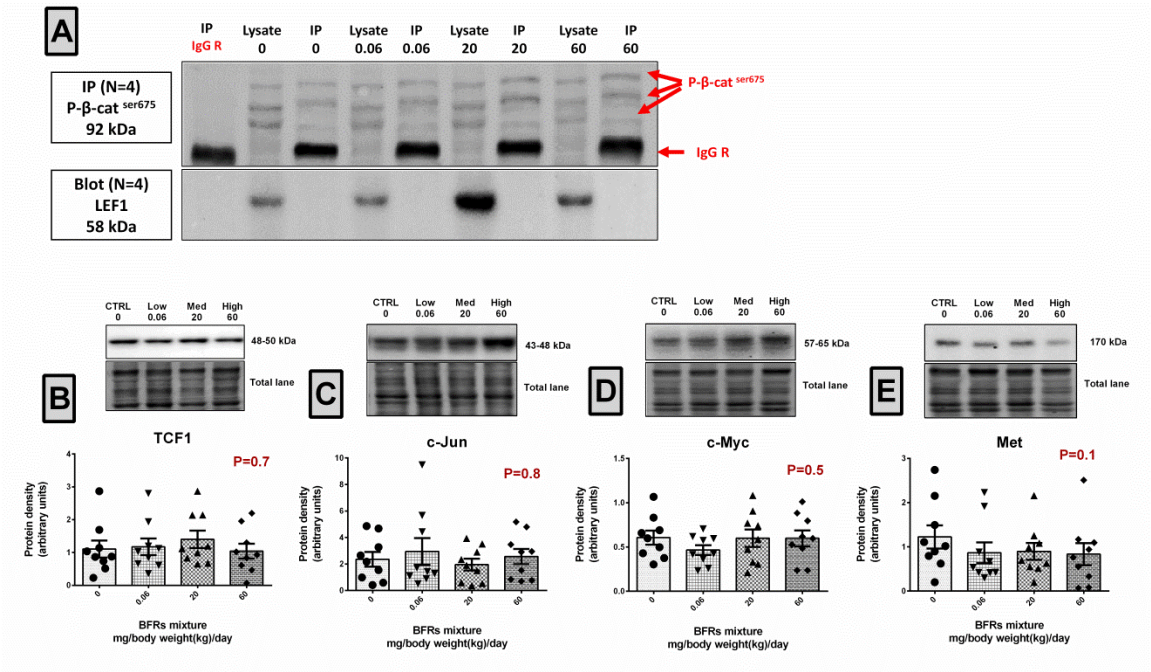
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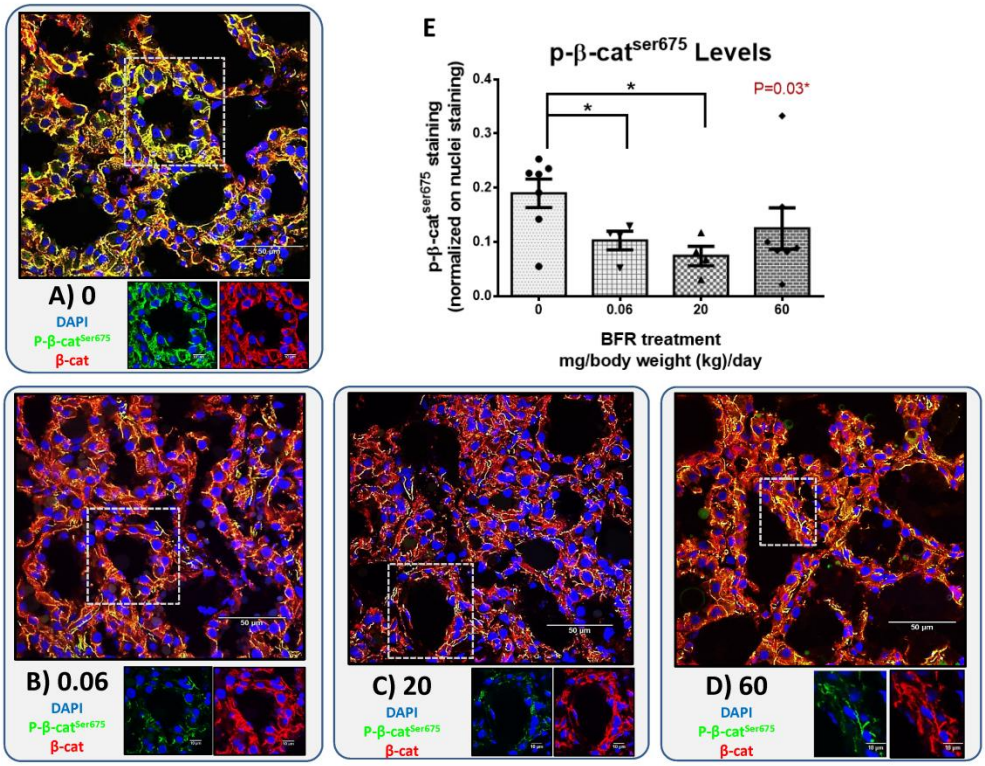
Figure 4)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β-cat
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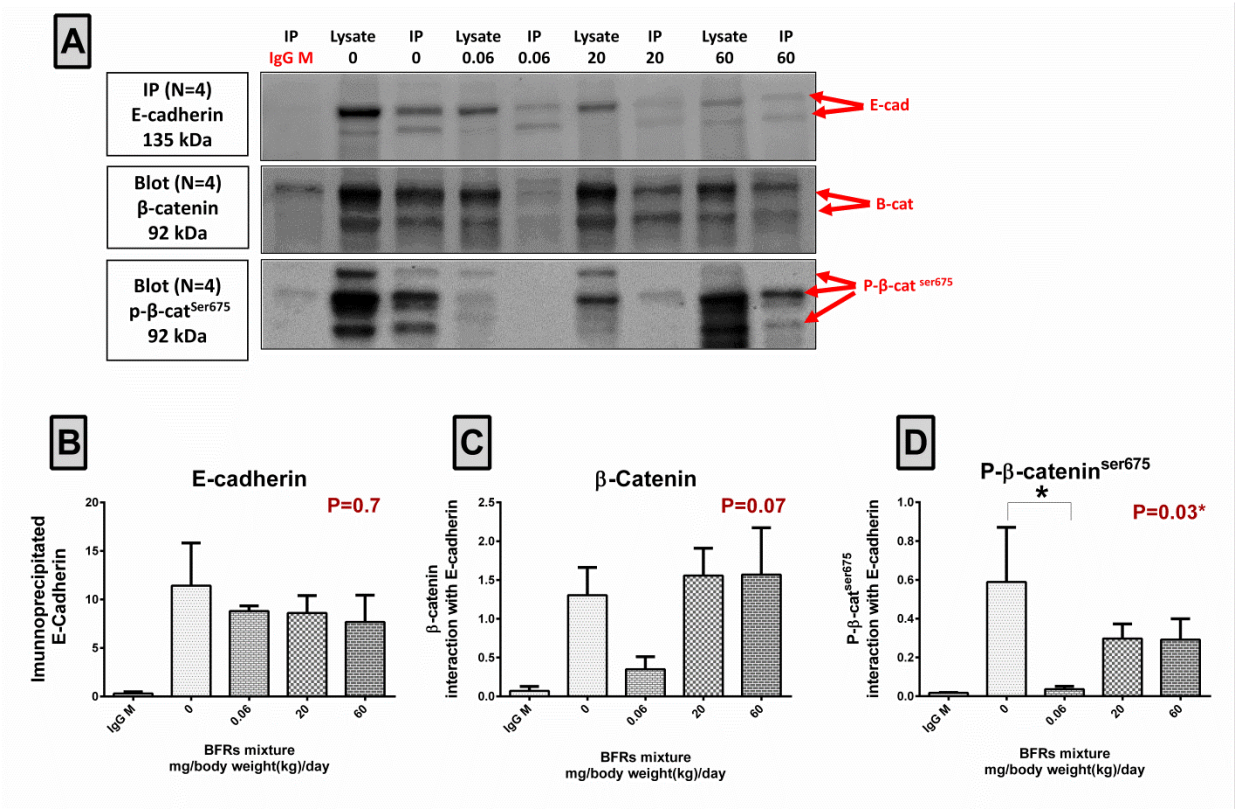
mg/body weight (kg)/day

Figure 5)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Blot (N=4) β-catenin 92 kDa
Blot (N=4) p-β-cat Ser675 92 kDa
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P-β-cat ser675
B
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C
β-Catenin
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β-catenin interaction with E-cadherin
D
P-β-catenin ser675
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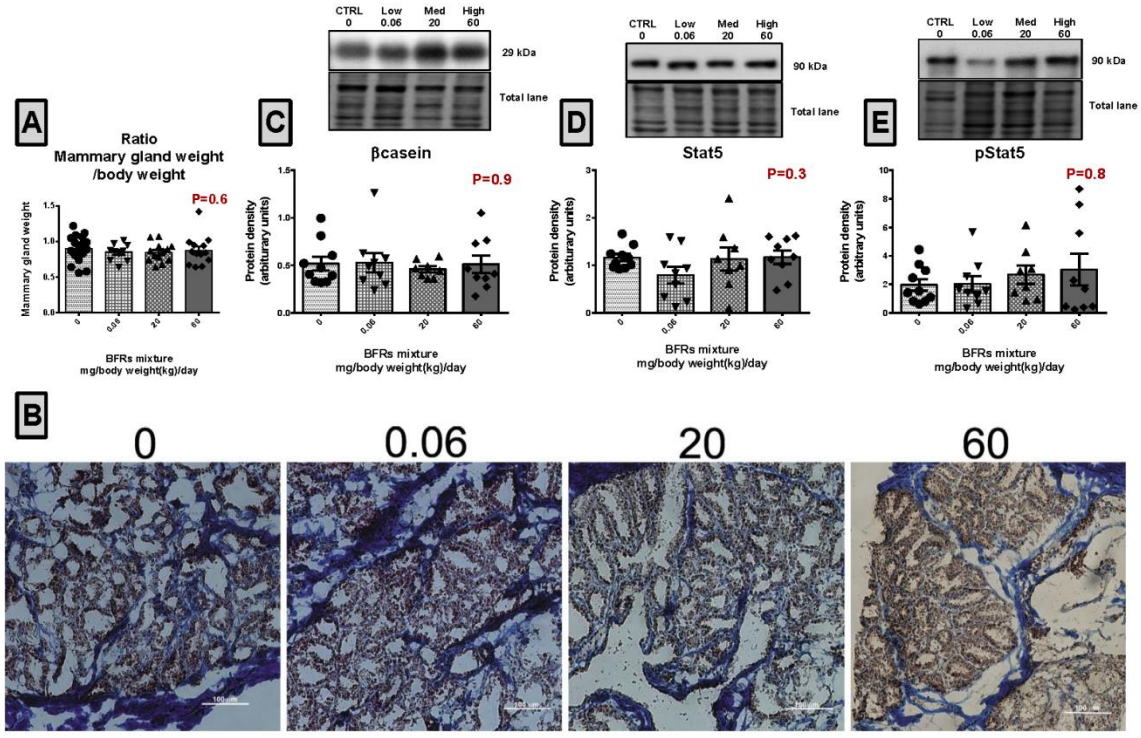
20
60
BFRs mixture mg/body weight(kg)/day

Sup figure 1)



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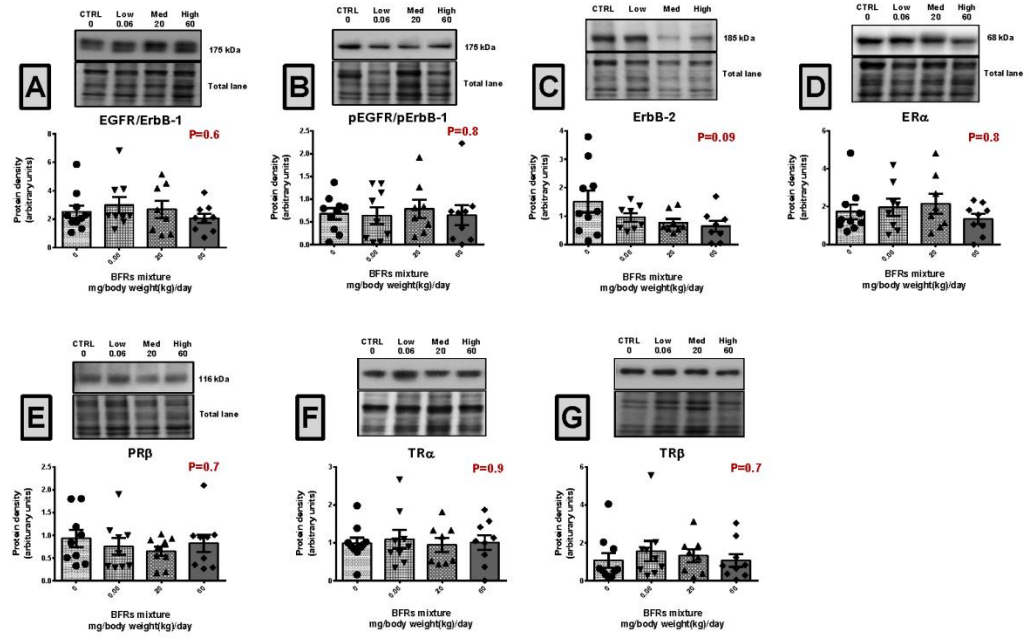

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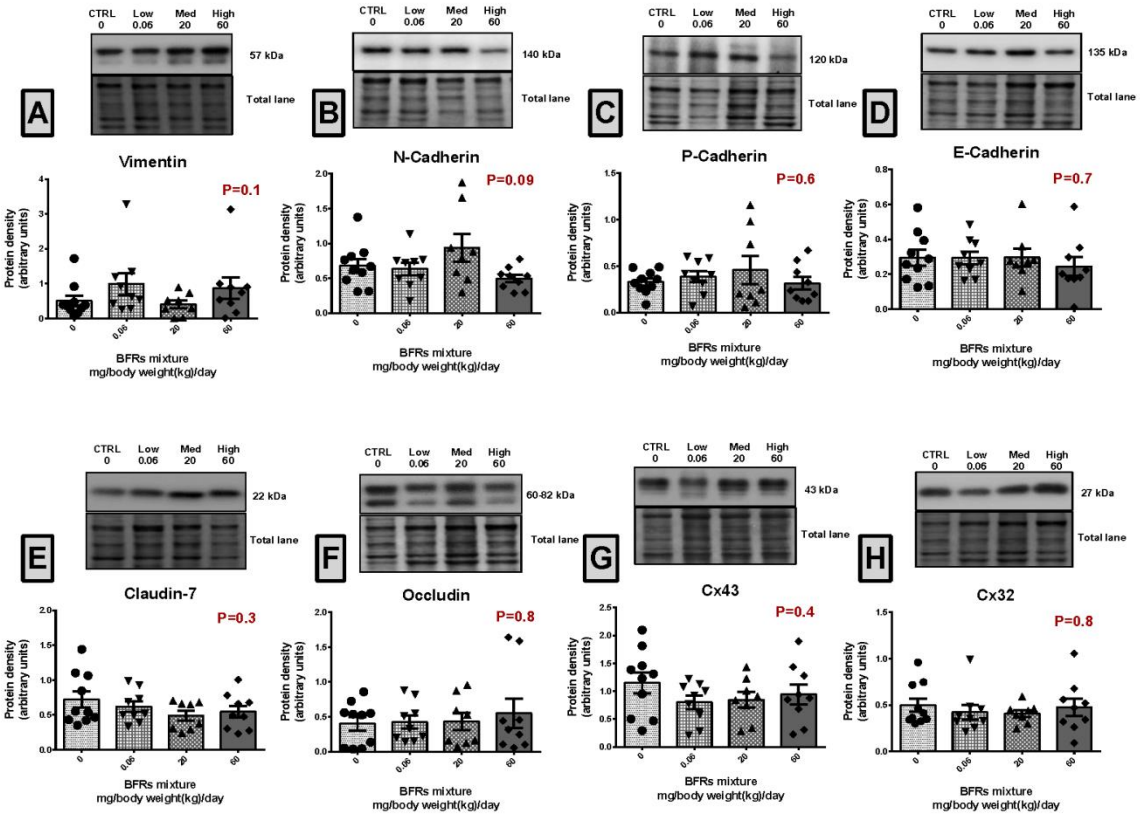
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Med 20
High 60
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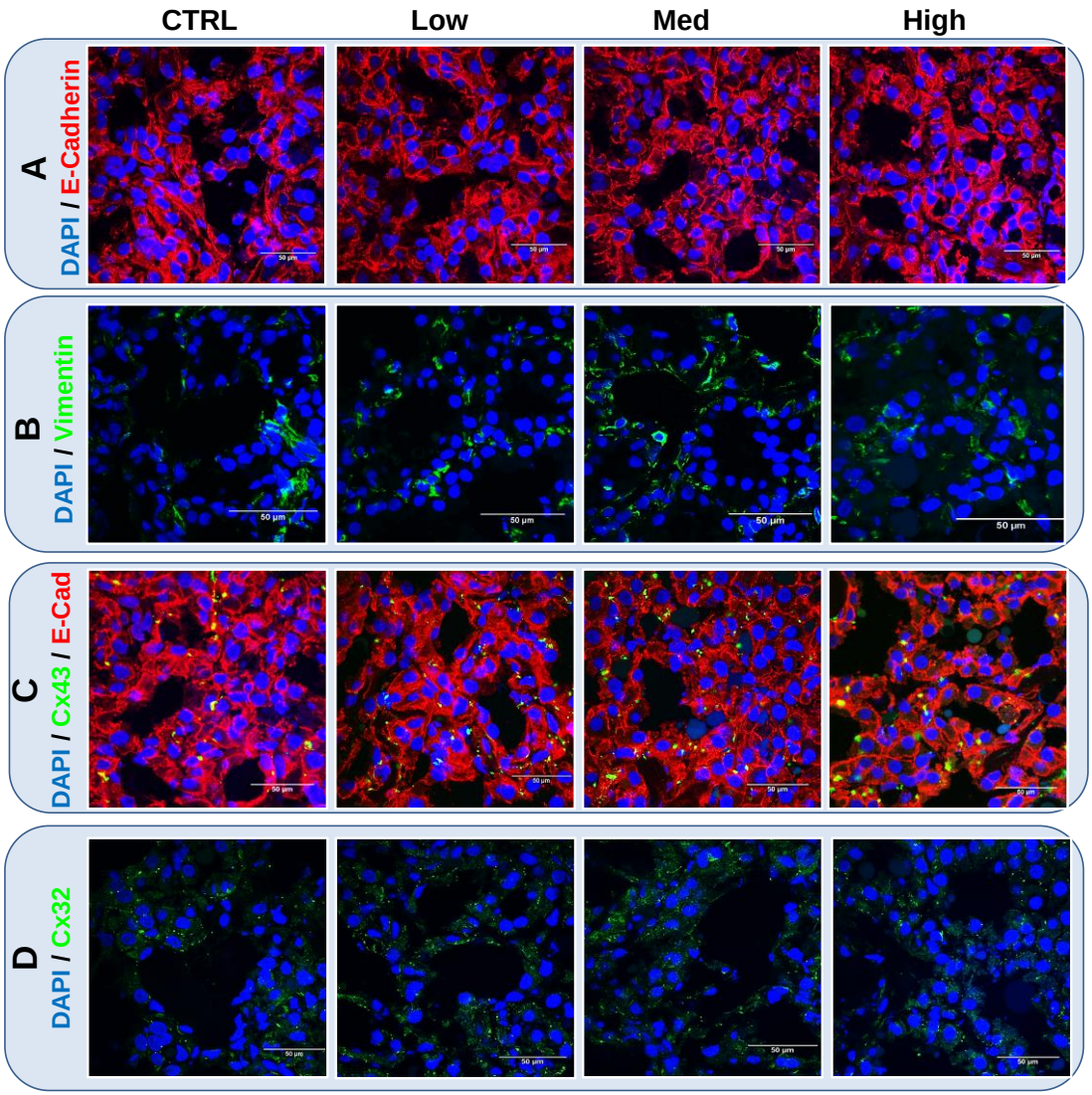
Total lane
Vimentin
P=0.1
Protein density (arbitrary units)
BFRs mixture mg/body weight(kg)/day
B
140 kDa
N-Cadherin
P=0.09
C
120 kDa
P-Cadherin
P=0.6
D
135 kDa
E-Cadherin
P=0.7
E
22 kDa
Claudin-7
P=0.3
F
60-82 kDa
Occludin
P=0.8
G
43 kDa
Cx43
P=0.4
H
27 kDa
Cx32
P=0.8

Sup Figure 4)


CTRL
Low
Med
High
A
DAPI / E-Cadherin
B
DAPI / Vimentin
C
DAPI / Cx43 / E-Cad
D
DAPI / Cx32
50 µm

Sup Figure 5)

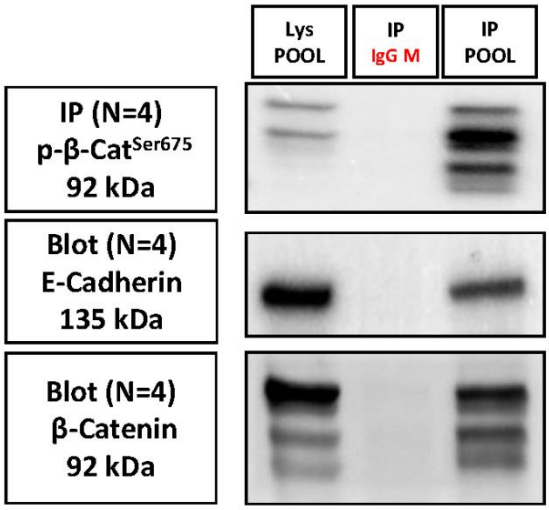

Lys
POOL
IP
IgG M
IP
POOL
IP (N=4)
p-β-Cat Ser675
92 kDa
Blot (N=4)
E-Cadherin
135 kDa
Blot (N=4)
β-Catenin
92 kDa

Table 1: Antibodies used for Western Blot analysis.			
Target protein	**Description/Host**	**Dilution**	**Catalogue number**
First antibodies			
β-catenin[1]	(L54E2) Mouse	1/2000	8480
E-Cadherin[1]	(4A2) Mouse	1/1000	14472
phospho-Stat5 (Tyr 694)[1]	Rabbit	1/1000	4322
Stat5[1]	Rabbit	1/1000	9363
EGF Receptor HER1/ErbB1[1]	(C74B9) Rabbit	1/500	2646
Phospho-β-Catenin (Ser675)[1]	(D2F1) Rabbit	1/1000	4176
Phospho-EGF Receptor (Tyr1068)[1]	(D7A5) XP Rabbit	1/1000	3777
GSK-3β[1]	(3D10) Mouse	1/1000	9832
N-Cadherin[1]	Rabbit	1/1000	4061
HER2/ErbB2	(29D8) Rabbit	1/1000	2165
Vimentin[1]	(D21H3) XP Rabbit	1/1000	5741
Phospho-GSK-3β (Ser9)[1]	(D85E12) XP Rabbit	1/1000	8213
Phospho-Akt (Ser473)[1]	(D9E) XP Rabbit	1/2000	4060
Akt (pan)[1]	(C67E7) Rabbit	1/1000	4691
Non-phospho (Active) β-Catenin (Ser45)[1]	Rabbit	1/1000	19807
c-Jun (60A8)[1]	(60A8) Rabbit	1/1000	9165
Met[1]	(D1C2) XP® Rabbit	1/1000	8198
c-Myc[1]	(D84C12) Rabbit	1/1000	5605
TCF1[1]	(C63D9) Rabbit	1/1000	2203
LEF1[1]	(C12A5) Rabbit	1/1000	2230
Phospho-CREB (Ser133)[1]	(87G3) Rabbit	1/1000	9198
Claudin-7[2]	Rabbit	1/1000	34-9100
Connexin 32[11]	Rabbit	1/1000	265-279
Connexin 43[11]	Rabbit	1/2500	C6219
P-cadherin[2]	6A9 Mouse	1/2000	MA1-2003
Occludin[10]	(H-279) Rabbit	1/500	sc-5562
β-casein[10]	(H-7) Mouse	1/500	sc-166520
PKAα[10]	(C-20) Rabbit	1/500	sc-903
p-Tyr 654-β-Catenin[10]	(1B11) Mouse	1/500	sc-57533
CREB[10]	(C21) Rabbit	1/500	sc-186
Anti-Estrogen Receptor alpha[9]	Rabbit	1/500	ab75635
Anti-Thyroid Hormone Receptor alpha[9]	Rabbit	1/500	ab53729
Anti-Thyroid Hormone Receptor beta[9]	Rabbit	1/500	ab196484
Secondary antibody			

Anti-rabbit IgG HRP-linked antibody[1]		1/5000	7074s
anti-mouse IgG HRP-linked antibody[1]		1/5000	7076s

[1] Cell Signaling, Beverly, MA, USA
[2] Thermo Scientific, Rockford, Illinois, USA
[3] VWR International, Ville Mont-Royal, QC, Canada
[4] Charles River Canada, St. Constant, Quebec, Canada
[5] Fisher Scientific, Burlington, ON, Canada
[6] Thermo Scientific, Mississauga, ON, Canada
[7] Cedarlane, Burlington, ON, Canada
[8] Millipore, Etobicoke, ON, Canada
[9] Abcam, Toronto, ON, Canada
[10] Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA
[11] Sigma-Aldrich, Oakville, Ontario, Canada
[12] Life Technologies/Invitrogen (Waltham, MA, USA)

Table 2: Antibodies used for immunofluorescent analysis			
Target protein	**Description/Host**	**Dilution**	**Catalogue**
First antibodies			**number**
E-cadherin[1]	(4A2) Mouse	1/50	14472
β-catenin[1]	(L54E2) Mouse	1/200	8480
Phospho-β-Catenin (Ser675)[1]	(D2F1) Rabbit	1/100	4176
Vimentin[1]	(D21H3) XP Rabbit	1/100	5741
Connexin 32[11]	Rabbit	1/100	265-279
Connexin 43[11]	Rabbit	1/500	C6219
N-cadherin[1]	Rabbit	1/100	4061
Second antibodies			
anti-rabbit IgG Fab2 Alexa Fluor 488[1]		1/1000	4412s
anti-mouse IgG Fab2 Alexa Fluor 647[1]		1/1000	4410s
anti-mouse IgG Fab2 Alexa Fluor 555[1]		1/1000	4409s
donkey anti-rabbit IgG antibody, Alexa Fluor® 568 conjugate[12]		1/1000	A10042

[1] Cell Signaling, Beverly, MA, USA
[2] Thermo Scientific, Rockford, Illinois, USA
[3] VWR International, Ville Mont-Royal, QC, Canada
[4] Charles River Canada, St. Constant, Quebec, Canada
[5] Fisher Scientific, Burlington, ON, Canada
[6] Thermo Scientific, Mississauga, ON, Canada
[7] Cedarlane, Burlington, ON, Canada
[8] Millipore, Etobicoke, ON, Canada
[9] Abcam, Toronto, ON, Canada
[10] Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA
[11] Sigma-Aldrich, Oakville, Ontario, Canada
[12] Life Technologies/Invitrogen (Waltham, MA, USA)

Table 3: Antibodies used for co-immunoprecipitation analysis				
Target protein	**Description/Host**	**Procedure**	**Dilution**	**Catalogue number**
E-Cadherin[1]	(4A2) Mouse	Immunoprecipitation	1/200	14472
Isotype control IgG mouse[1]	G3A1	Immunoprecipitation	1/400	5415
E-Cadherin[1]	(4A2) Mouse	Blotting	1/1000	14472
Mouse IgG[1]	Alexa Fluor 647	Secondary antibody for E-cadherin	1/5000	4410
β-catenin[1]	(L54E2) Mouse	Blotting	1/2000	8480
Mouse IgG[12]	Alexa Flour 488	Secondary antibody for β-catenin	1/5000	A11001
Phospho-β-Catenin (Ser675)[1]	(D2F1) Rabbit	Blotting	1/1000	4176
Rabbit IgG[9]	HRP-conjugated Veriblot for IP	Secondary antibody for Phospho-β-Catenin (Ser675)	1/5000	ab131366
Phospho-β-Catenin (Ser675)[1]	(D2F1) Rabbit	Immunoprecipitation	1/200	4176
Isotype control IgG rabbit[1]	DA1E	Immunoprecipitation	1/400	3900
Phospho-β-Catenin (Ser675)[1]	(D2F1) Rabbit	Blotting	1/1000	4176
Rabbit IgG[1]	Alexa Fluor 488	Secondary antibody for Phospho-β-Catenin (Ser675)	1/5000	4412
LEF1[1]	(C12A5) Rabbit	Blotting	1/1000	2230
Rabbit IgG[9]	HRP-conjugated Veriblot for IP	Secondary antibody for LEF1	1/5000	ab131366

[1] Cell Signaling, Beverly, MA, USA
[2] Thermo Scientific, Rockford, Illinois, USA
[3] VWR International, Ville Mont-Royal, QC, Canada
[4] Charles River Canada, St. Constant, Quebec, Canada
[5] Fisher Scientific, Burlington, ON, Canada
[6] Thermo Scientific, Mississauga, ON, Canada
[7] Cedarlane, Burlington, ON, Canada
[8] Millipore, Etobicoke, ON, Canada
[9] Abcam, Toronto, ON, Canada
[10] Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA
[11] Sigma-Aldrich, Oakville, Ontario, Canada
[12] Life Technologies/Invitrogen (Waltham, MA, USA)

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3 Conclusion and perspectives

Mammary gland development resembles some cellular aspects that are also hallmarks of cancer. For instance, there are phases of extensive cell proliferation (puberty and pregnancy), cell differentiation (puberty and lactation) and cell apoptosis and parenchyma remodeling (involution) across the development. Moreover, during the entire reproductive life of mammals, these phases of proliferation-differentiation-apoptosis are repeated. In cancer, these events are typically dysregulated. Thus, understanding how cell-cell interactions are regulated and contribute to these processes is very beneficial to better understand their contribution to pathogenesis.

Together, our results have shown that proteins from gap, tight and adherens junctions are expressed in a stage-dependent manner and form dynamic nexuses that vary across mammary gland development. These results have elucidated the plastic nature of mammary glands structure in context of intercellular junctions, as cell-cell interactions were modified following the requirements of the tissue. Although the identified protein-protein interactions among intercellular junctions are an important mode of regulation, this stage dependant remodeling is perhaps regulated by changes in the hormonal balance. This study established a basic mechanism of junctional proteins interplay across the post-natal development. This knowledge can help scientists in order to better identify the bypassed and corrupted targets in abnormal development as well as breast cancer.

In accordance, our results have demonstrated that a low environmentally relevant dose of a mixture of brominated flame retardants, with an endocrine disruptive property, has influenced junctional proteins' expression and interaction. Although these dysregulations did not seem to impact the mammary gland function, i.e. the lactation, more studies are required to determine the long-term effects associated to the observed effects. Defects in developmental pathways can contribute to the initiation and progression of cancer.

Altogether, the outcome of this study illustrated the findings on junctional nexus remodelling and their corruption upon BFRs administration. Involvement of different junctional proteins at each certain stages of post-natal development requires rapid plasticity in assembly, disassembly and positioning of junctions. BFRs exposure presumably linked to hormonal unbalance has had influenced nexus assembly in lactation.

Unraveling the underlying regulators of junctions remodeling at each stage of development is certainly required in order to identify the corrupted mechanisms and factors associated to breast anomalies and cancer. Although our research brought important insights on the junctional nexus in the mammary gland and introduced them as potential targets for BFRs, not all questions were answered. Investigating more potential targets, the complete signaling cascades related to the identified ones, and the long term effect of BFRs in breast anomaly and cancer remain to be elucidated.

The differential needs for cell-cell communication during mammary gland development and function

Three Cxs exhibited overlapping windows of expression during pregnancy and lactation. Physical interaction, showed by our immunoprecipitation assays, between Cx26-Cx30 and Cx26-Cx32 at cell membrane during pregnancy and lactation (Figure 13) suggest the formation of heteromeric connexons and/or heterotypic gap junction channel at these stages. The formation of heteromeric/ hemichannels made of Cx26-Cx32-Cx30 and their presence in the same GJs plaque during lactation were previously shown (Locke, Perusinghe et al. 2000) (Locke, Jamieson et al. 2007). Cxs forming GJs allow the diffusion of molecules and ions ≤1 kD mediating cell-cell communication. Studies have shown that small metabolites, second messengers, sodium, potassium, calcium, AMP, GMP, ADP and ATP can pass through GJs. It has been demonstrated that Cxs composition can directly influence on GJs channels' conductance and permeability (Bevans, Kordel et al. 1998) (Ayad, Locke et al. 2006) (Locke, Perusinghe et al. 2000, Locke, Stein et al. 2004). In addition Cxs are implicated in hemichannels formation (Jiang and Gu 2005); connexons hemichannels are implicated in intercellular signalling and play different role than GJs (Goodenough and Paul 2003). It has been shown that Cx43 hemichannel can induce cell proliferation through mediating ATP release and intracellular Ca2+ concentration (Song, Liu et al. 2010) (Pearson, Dale et al. 2005). Our data indicated that four Cxs (Cx43, Cx26, Cx30 and Cx32) are differentially expressed across mammary gland development. It is presumed that through the formation of different hemichannels and GJs channels, the four Cxs are involved in differential conductance and permeability. Thus, it can be presumed that differential expression of Cxs is linked to the selective passage of the molecules that need to pass through adjacent cells and vary from stage to stage across the development. In addition, the observed overlapping expression and interaction between Cxs during late pregnancy and first trimester of lactation suggest a potential compensatory mechanisms among these Cxs which can explain why conditional knockout of Cx26 during mid and late

pregnancy had no effect on lobuloalveolar development (Stewart, Plante et al. 2014) (Bry, Maass et al. 2004).

Cxs expression is not only tissue dependant, but also variable depending on the stage of development, suggesting that their expression is being tightly controlled and regulated. Thereby, finding the regulatory mechanisms beyond Cxs precise up- or down-regulation during pregnancy or lactation are crucial to further understanding their stage-dependent function during mammary glands development. In addition, more *in vitro* research is required in order to investigate the GJs formation, selective permeability and connexons involvement in signaling.

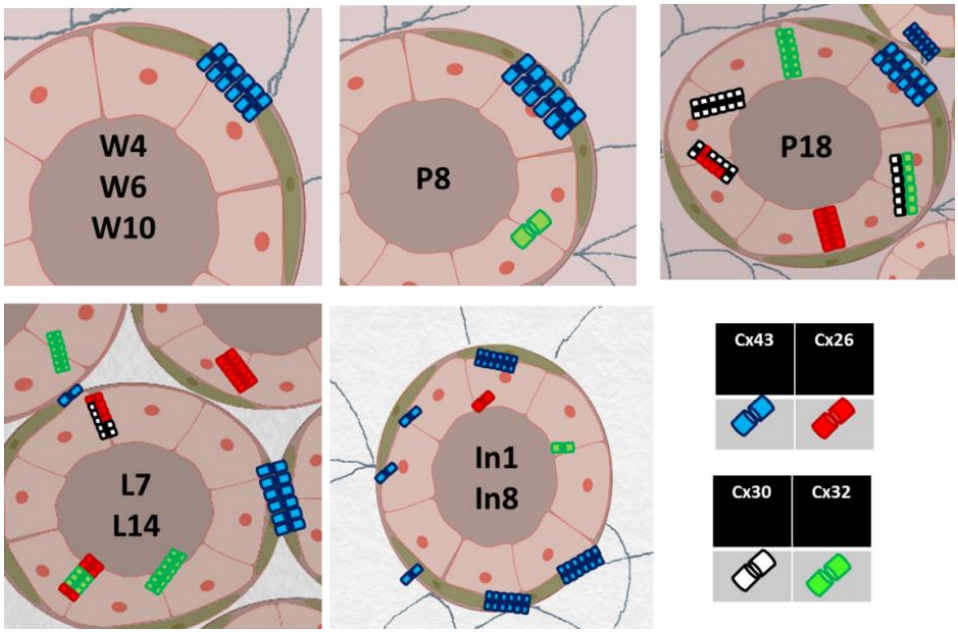

Figure 13) Schematic view of Connexins (Cxs), Cx43, Cx26, Cx30 and Cx32, across the mammary gland development. Protein expression, localization and interaction between Cxs are summarized for different stages of development; before puberty (W4), during puberty (W6), adult (W10), Pregnancy day 8 and 18 (P8, P18), lactation day 7 and 14 (L7 and L14), Involution day 1 and 8 (In1 and In8) (Dianati, 2017 unpublished).

Our outcomes showed that Gap, tight and adherens junctions demonstrated differential expression pattern at gene and protein levels across the post-natal mammary gland development. Although differential expression of Cxs and Claudins have been shown before (Locke, Perusinghe et al. 2000) (Locke, Jamieson et al. 2007) (Blackman, Russell et al. 2005, Blanchard, Watson et al. 2006), the

window of study did not entirely cover all stages of development or only gene expression was studied. Similarly, although AJs components' implication in breast cancer has been known and widely studied for years, our results are the first to show their profile of expression across the development.

Junctional proteins interplay within the epithelium forming the different nexus

Cxs are not only involved in GJs formation but are also implicated in cell signaling via interacting with several binding partners, including cytoskeletal elements, enzymes, kinases as well as TJ and AJs components (Talhouk, Mroue et al. 2008). Reciprocal interaction between Cxs and AJs and TJs components in different tissues and their functions on each other's assembly are thoroughly summarized in these reviews (Duffy, Delmar et al. 2002) (Derangeon, Spray et al. 2009). Although numerous reports established the functional involvement of ZO-1, E-cadherin, N-cadherin, β-catenin and p120-catenin with GJs in different tissues (Giepmans 2004) (Jongen, Fitzgerald et al. 1991, Ale-Agha, Galban et al. 2009), little is known in mammary glands. There is one *in vitro* study which shown that the recruitment of α-catenin, β-catenin and ZO-1 in to the GJs complexes increased the functionality of GJs and differentiation process in murine mammary cell lines (Talhouk, Mroue et al. 2008), indicating the importance of junctional interplay in breast development.

Consistently, we have demonstrated that junctional proteins interplay within the epithelium of the mammary gland (Figure 14), proposing that they regulate each other's function or contribute to one another's stabilization. Cx43 differentially interacted with components of TJs and AJs during mammary gland development while Cx32 only intermingled with AJs components during lactation. Obtaining these results could not establish the direct protein-protein interaction between the identified binding partners but their residence at the same junctional nexus. Further research is required in order to pinpoint the direct partners or other mediators linking these protein interactions.

Intercellular junctions are required for preserving tissue integrity, structure, homeostasis and polarity (Saito, Tucker et al. 2012) (Keiper, Santoso et al. 2005). In mammary glands, intercellular junctions has been shown to provide the interactions between luminal cells and between myoepithelial cells (i.e. homocellular interactions) that are required for the maintenance of tissue integrity (Runswick, O'Hare et al. 2001, Gudjonsson, Adriance et al. 2005). However, the involvement of heterocellular junctions between luminal and myoepithelial cells in mammary glands development was not known. We have observed that Cx43 mainly localizes between myoepithelial cells, as well as between myoepithelial and luminal cells, and little expression between luminal cells. As a result, the identified

nexuses (Cx43/E-cadherin/Claudin-7 and β-catenin) likely link the luminal and the myoepithelial cells together and presumably serve a role in tissue integrity (Figure 14). Moreover, the interaction between these heterocellular binding partners is stronger at late pregnancy. It has been shown that heterocellular interactions mediate cell differentiation and production of β-casein (Barcellos-Hoff, Aggeler et al. 1989) and recruit α-catenin, β-catenin and ZO-2 in to the GJs made of Cx43 and Cx32 (Talhouk, Mroue et al. 2008). Thus, it can be easily speculated that Cx43/E-cadherin/Claudin-7 and β-catenin interaction between heterocellular compartments and another identified nexus, Cx32/E-cadherin and β-catenin, between luminal cells serve the stage dependant functions (Figure 14). More research is required in order to elucidate these nexuses functions in milk signaling pathways and adhesion throughout lactation.

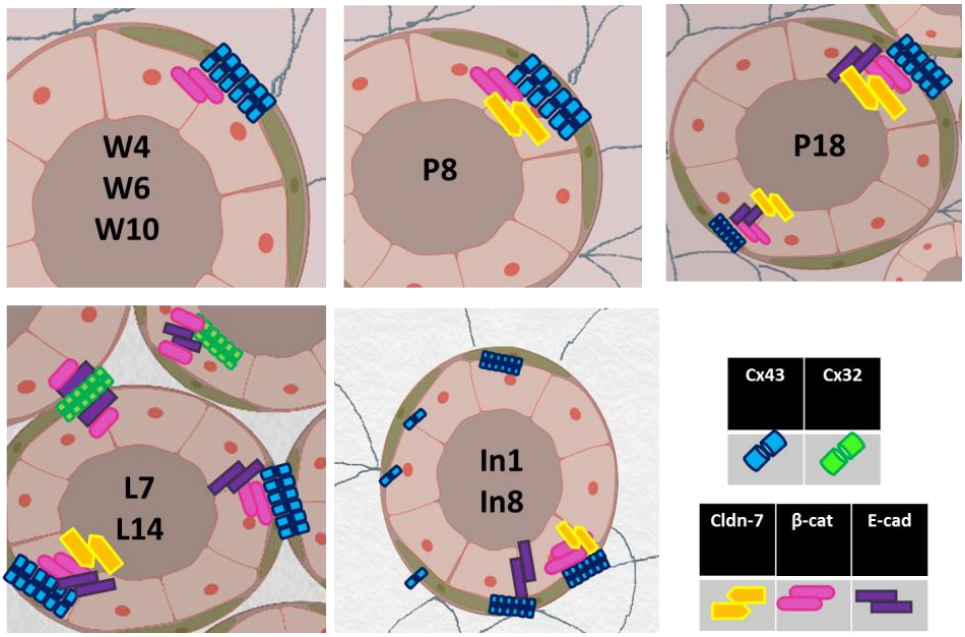

Figure 14) Schematic overview of differential junctional nexus across the mammary gland development; Protein expression, localization and interaction between Cxs are summarized for different stages of development; before puberty (W4), during puberty (W6), adult (W10), Pregnancy day 8 and 18 (P8, P18), lactation day 7 and 14 (L7 and L14), Involution day 1 and 8 (In1 and In8). Cx43 interacts with TJs and AJs components, Claudin-7, E-cadherin and β-catenin. Cx32 intermingle with AJs components, E-cadherin and β-catenin (Dianati, 2017 unpublished).

In spite of the fact that our findings contributed a lot to the understanding of cell-cell interactions via junctions across the mammary gland development, this is just the beginning of future

research on identification of the sequential arrangement of these proteins at cell membrane. Future research will aim to elucidate which protein(s) or mechanism(s) drag and recruit junctional proteins within each nexuses. Further experiments are essential to unravel whether GJs, TJs and AJs regulate each other's function and/or stabilization at the cell membrane of mammary glands at those specific stages as previously reported (Derangeon, Spray et al. 2009) (Talhouk, Mroue et al. 2008) .

The outcome of our study elucidated that Cxs demonstrated various permanent and stage-dependant interactions with certain components of TJs and AJs. However, the importance of these nexuses in mammary gland structure and function remained to be elucidated. Understanding the dynamic of the junctional nexus formed by GJs, TJs and AJs components during mammary gland development is important to better understand mammary gland organogenesis and identify the abrogated mechanisms associated to abnormal development and breast cancer.

Junctional proteins in breast cancer initiation and progression

Dysregulation of intercellular junctions in epithelial compartments are associated with many types of cancers including breast cancer. Cell-cell interactions via junctions are not static and remodel based on the requirements of the tissue, such as proliferation and apoptosis which recapitulate different features of cancer. For instance E-cadherin loss of expression and mutation has been linked to tumor invasion and poor prognosis (Berx, Cleton-Jansen et al. 1995, Berx, Cleton-Jansen et al. 1996, Berx and Van Roy 2001) (Conacci-Sorrell, Zhurinsky et al. 2002). In addition E-cadherin/catenin complex preserves the tissues integrity functioning as a tumor suppressor (Berx, Cleton-Jansen et al. 1995, Berx, Cleton-Jansen et al. 1996, Berx and Van Roy 2001) (Conacci-Sorrell, Zhurinsky et al. 2002). Loss of membranous β-catenin is correlated with significant worse outcome in patients with breast cancer (Dolled-Filhart, McCabe et al. 2006). Another type of dysregulation in junctional proteins occurs during Epithelial Mesenchymal Transition (EMT). EMT which is essential during organogenesis as well as cancer is a remodeling of adherent epithelial cells to the less attached mesenchymal types. This mostly occurs via switch within intercellular junctions , i.e. the switch from E-cadherin to N-cadherin and vimentin (Diepenbruck and Christofori 2016).

Moreover proper mislocalization of Occludin and Claudin-1 is important in providing the leaky junctions leading to the shuttle of luminal growth factors to basolateral surface (Brennan, Offiah et al.

2010) and down-regulation of Occludin, Claudin-1, -4 and -6 and up-regulation of Claudin-3 and -7 are reported in breast cancer (Brennan, Offiah et al. 2010).

Impaired GJs is essential in early stages of tumorigenesis, while functional GJs is essential for invasion, intravasation and extravasation of tumor cells (Czyz 2008) (El-Saghir, El-Habre et al. 2011). Thus Cxs play a paradoxical role as a tumor enhancer or suppressor depending to the stage of carcinogenesis and metastatic or primary tumors (Czyz 2008). Dysregulation of Cxs is associated with developmental defects, breast cancer (Lee, Tomasetto et al. 1992, Carystinos, Bier et al. 2001, Kanczuga-Koda, Sulkowski et al. 2006, Naus and Laird 2010) and metastasis (Carystinos, Bier et al. 2001).

Cxs roles in organogenesis and cell-cell communication via GJs-dependent function create challenges on targeting it for developing new therapeutics anticancer drugs. Thereby targeting Cxs-interacting proteins can be considered as a more secure strategy to reduce the side effects associated with GJs loss. The outcome of this research introducing multiple Cxs interacting proteins during normal development has contributed a lot to the field of Cxs independent GJs function in breast tumorigenesis. The observed interaction among Cxs and other junctional proteins and the nexus remodeling across the development links their function to one another and thus can be the potential corrupted target in breast cancer.

It is assumed that the junctional nexus that is permanently present throughout TEBs expansion, ductal branching, cell proliferation, alveologenesis and apoptosis harbors similar regulators and all together serve common functions during the gland development. However, stage dependent nexus will pinpoint individual stage dependent role and regulation and can likely be the corrupted markers which differ between different types of cancer.

Regulation of the junctional nexus during mammary gland development

During mammary glands development, cell-cell interaction varies in response to physiological changes. Junctions' differential gene/ protein expression and their remodeling across the development suggest the precise and distinct regulators. We have already discussed the possibility of their regulations through protein-protein interactions with their identified binding partners at cell peripheries. Another important factor that could be implicated in regulation of junctions throughout

development is the systemic hormones and paracrine factors which are essential for gland development, vary at each stage and have been linked to the junctions remodelling in the other tissues, as well as mammary glands.

As suggested in Figure 15, junctional proteins remodeling can be tightly regulated by hormones and paracrine factors. For instance, at puberty and in adulthood, the levels of Cx43 remained high, suggesting the involvement of E2, P4 or GH through IGF-I in its regulation. By contrast, Cx43 is down-regulated during lactation while Cx32 is up-regulated, indicating that PRL secretion or drop in P4 or their synergic effect can negatively and positively regulate expression of different Cxs. Previous research showed Cx43 augmentation after E2 peak and its reduction upon P4 withdrawal in the endometrium (Petrocelli and Lye 1993). Interestingly, at the end of pregnancy, Cx26, Cx30, Cldn-7, E-cadherin and β-catenin were all up-regulated, suggesting a common mechanism of regulation, likely through P4 and/or PRL. Previous studies in uterine and sertoli cells are supporting the possibility of hormonal regulation of these proteins (Satterfield, Dunlap et al. 2007). Similarly, P-cadherin peak of expression in adulthood and early pregnancy suggest a positive regulation via P4 and possible negative control via PRL and PL. Accordingly, it has been previously shown that AJs can be regulated by P4 (Satterfield, Dunlap et al. 2007) (Sluka, O'Donnell et al. 2006).

Another remarkable change between pregnancy and lactation was the change of localization for Claudin-7 from basolateral to mostly apical sites during late pregnancy. As Claudins are the charge and size selective members of TJs, these results suggest that Claudin-7 is the candidate transmembrane protein responsible for the switch in selective permeability of TJs from pregnancy to the lactation. P4, PRL, glucocorticoids and placental lactogen were previously assigned to be involved in TJs closure upon lactation for prevention of milk leakage (Nguyen, Parlow et al. 2001). Thus, it is highly probable that these hormones regulate Claudin-7 localization shift. Although our results suggest the probability of hormonal regulation of junctions' expression, localization and their residence in the nexus, further *in vitro* research followed by *in vivo* murine models are requisite to ascertain the involvement of different hormones in the identified nexus formation and remodeling.

Altogether, we've shown that junctional proteins are differentially expressed and reside in different nexuses, likely in order to respond to physiological changes which argue for a potential effect of hormones on intercellular junctions. Looking at the identified junctional nexuses across the development (Figure 14), it is observable that two stages of development, late pregnancy and lactation,

acquired the most junctional interplay and nexus remodeling. The most noticeable junctional nexus present at late pregnancy is comprised of Cx43, E-cadherin, β-catenin and Claudin-7. While this nexus is present but less abundantly, another nexus consisting Cx32, E-cadherin and β-catenin was predominantly expressed during lactation (Figure 14). The structural change from a tissue containing ducts and alveoli to the one fully comprised of alveolar cells and acini are mediated by hormones and paracrine factors (Figure 15). Thus, the switch in nexuses present from pregnancy to lactation might also be driven by hormones. Since the implication of more than one hormonal driver is probable at certain stages of mammary gland development (Figure 15), further research is required to unravel the main trigger of each individual gene/protein expression.

An environmentally relevant mixture of brominated flame retardants affects junctional proteins

Our results so far, demonstrated the differential cell-cell interaction across the development and suggested that junctional proteins can be regulated, at least in part, by hormones. More importantly, our results introduced lactation as one of the most variable stages in terms of nexus remodeling, which could be susceptible to hormonal unbalance. Thereby, we next wanted to analyze the effects of a mixture of the potential EDs BFRs in samples from rats in lactation.

Although the environmental relevant mixture of BFRs resembling house dust in North America had EDs activities *in vitro* (Lefevre, Wade et al. 2016), our results showed that BFRs do not have major effects on hormonal regulatory functions, at least for the analyzed pathways. It should be noted that the pups that have been nursed by these dams were also assessed (Tung, Yan et al. 2016) demonstrating that BFRs had not any impact on litter size. Similarly, ductal branching and histology of the glands from the pups at later stages (post-natal day 21 (PND21), PND46 and PND208) did not show any defects (see publication in preparation annexed).

Consistent to our previous conclusion concerning the importance of cell-cell interaction at each stage of development, including lactation, as well as the potential effect of hormones on junctional nexus remodeling, we then assessed BFRs effect on junctional proteins expression, localization and interaction. Although, we did not evaluate all the intercellular junctions components, our data established interesting findings regarding BFRs effect on junctional proteins expression and interaction. The down-regulation of p-β-cat^{Ser675} protein, confirmed by both western blot and immunofluorescence

techniques, along with significant reduction of p-β-cat^{Ser675}/E-cadherin interaction at low environmentally relevant dose of BFRs confirmed the potential susceptibility of junctional proteins to hormonal unbalance. Although, we have shown that p-β-cat^{Ser675} reduction is likely PKA-dependent, as it was concomitant with a down-regulation of p-CREB, the long terms potential threat rising from these shortcomings requires further research.

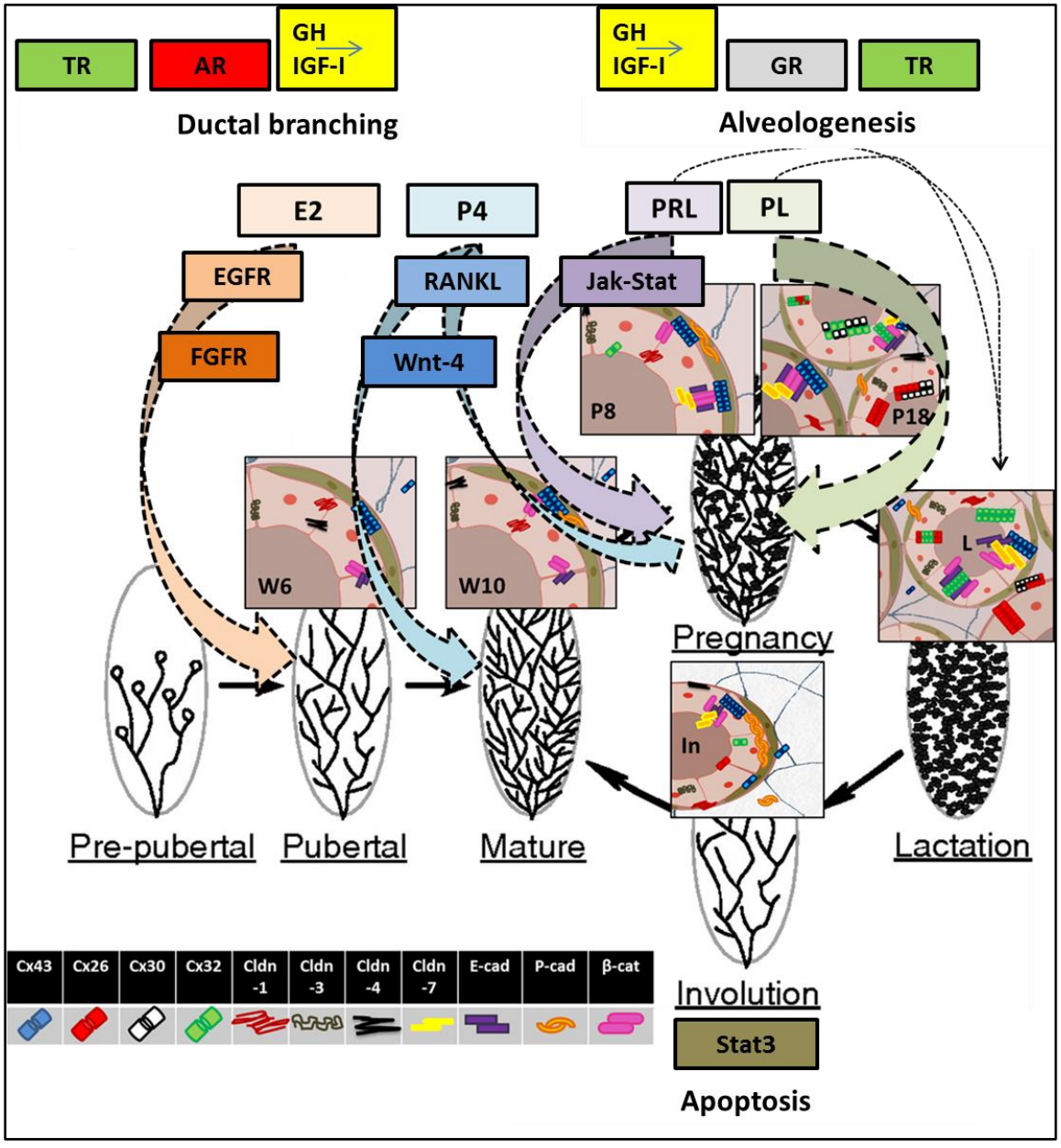

Figure 15) Junctional proteins remodeling can be regulated by hormones and growth factors. Gene, protein levels and protein-protein interaction was changed at different stages of development. This scheme suggests that cell-cell interaction remodeling can be through hormonal and paracrine factors stimulation (adapted from (Ben-Jonathan, Chen et al. 2009)).

Interestingly, perinatal exposure to low dose BFRs at the puberty time (PND46) also demonstrated an effect on junctional protein level. Indeed, at PND46, E-cadherin expression was down-regulated, further confirming that BFRs exposure targets cell-cell interaction proteins (annexe). Although BFRs targeted two different junctional proteins, these different effects can be simply explained. Lactation (dams) and puberty (pups) are two critical stages of development which are orchestrated by different hormones (Figure 15). Moreover, the structure of the gland and the physiological role of the gland also differ (publication 1). Thus, it can be speculated that either BFRs exposure perturbs diverse hormones or paracrine factors, and thus different signaling pathways are affected, or BFRs perturbs the same hormones, which have different effects depending on the stage. Part of this answer can be in the fact that the low dose of BFRs down-regulated the expression of the thyroid receptor α in mammary glands at puberty (annexe), but not in dams. Concluding this observation require further research as our results are in contrast with research which has reported the importance of TRs in lobuloalveolar development and no role in ductal branching (Vonderhaar and Greco 1979) (Borellini and Oka 1989). Whether the prenatal exposure to BFRs could perturb this receptor during puberty or this receptor really functions through ductal branching remain to be elucidated.

As the low dose effect is common for toxicants with endocrine activity, the observation of the low dose effects in mammary glands both during lactation in dams (publication 3) and at puberty in pups exposed during the perinatal period (annexe), established that a low environmentally relevant dose of BFRs influences mammary glands. The different effects between pups and dams, demonstrated that BFRs exposure abrogates glands signaling in various ways. Understanding of the complete signaling pathway and the long term effects associated to these anomalies yet remained to be determined.

General remarks

- Breast cancer is characterized by dysregulation of processes critical for mammary glands development, such as extensive cell proliferation at the time of puberty and pregnancy and apoptosis at the time of involution.
- Although intercellular junctions have been linked to breast cancer, their differential expression pattern at gene and protein level was not known. Our study is the first evaluation of AJs across the post-natal gland development and a broader confirmatory analysis on GJs and TJs expression pattern. These data are essential in order to unravel the abrogated mechanisms in pathology and breast cancer.
- Junctional proteins abrogation is linked to breast cancer
- Junctional proteins can't be directly targeted as cancer therapeutic targets because they serve multiple roles in preserving the gland structure, homeostasis and cell-cell interaction
- Junctional proteins interaction can be a compensatory safer therapeutic target since their interplay in different tissues are linked to one another's regulation, function and stability at cell peripheries in some tissues
- Our study is the first to demonstrating that GJs components in mammary glands, Cx43 and Cx32 interact with components of TJs and AJs and more importantly that these interactions are stage dependent. The state of these protein-protein interactions in different types and grades of breast cancer needs to be identified in order to determine the abrogated mechanisms and their potential therapeutic effect.
- Our research is also the first to demonstrate the susceptibility of intercellular junctions to a low environmentally relevant dose of a mixture of BFRs.
- The observed effects of BFRs at different stages of development were consistently on AJs, introducing AJs as the most vulnerable junctions.
- Perturbation in AJs is highly correlated with breast cancer. AJs are known to be the first junctions present at cell peripheries to bring cell membranes closer, thus allowing the localization of GJs and TJs at the closely associated areas between cells. Thus, understanding the long term anomalies associated to these shortcomings can contribute a lot to the field of breast cancer.

4 Annexe

Perinatal Exposure to Brominated Flame Retardants Suppresses E-cadherin and Thyroid Hormone Receptor α Expression in Mammary Glands at Puberty

Titre en française: Une exposition périnatale à un mélange de retardateurs de flammes bromés (BFRs) inhibe E-cadhérine et THRα à la puberté, dans les glandes mammaires

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Authors' contribution;

Mélanie Lavoie did most of the experiments, wrote the draft of the manuscript and contributed to the final preparation and revision of the manuscript.

Elham Dianati contributed to study design as well as in troubleshooting and optimisation of protocols, performed all the animal sacrifice and tissue harvesting, helped in the supervision of the first author, generated the data presented in figures 1 and 2A-F, and contributed the final preparation and revision of the manuscript.

Mike Wade, Barbara Hales and Bernard Robaire contributed to design of animal study and to the final preparation and revision of the manuscript.

Isabelle Plante contributed to the design of the animal study, has directed the project and contributed to the preparation and revision of the manuscript.

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Abstract

Brominated flame retardants (BFRs) are chemicals widely used in consumer products including electronics, vehicles, plastics and textiles to reduce ignition rates. Even though usage of BFRs has been restricted in many countries, mainly due to their persistence, bioaccumulation and potential toxicity, human exposure continues as BFRs are still released from existing items. Also, the role of BFRs in breast development and cancer, as well as the mechanisms implicated in the compound's toxicity, remain poorly understood. This project aimed to assess the effects of a perinatal exposure to BFRs on mammary gland development and breast cancer, and to determine mechanisms involved in BFRs' toxicology. Female rats were exposed through diet to an environmentally relevant mixture of BFRs two weeks prior to mating, during gestation and during lactation. Female offspring were sampled at critical stages of mammary gland development including post-natal day 21 (PND21), 46 (PND46) and 208 (PND208). BFRs treatment had no significant effect on total body weight or mammary gland weight nor did it affect the epithelial surface area or the elongation of the lactiferous ducts. The protein expression of E-cadherin, a protein involved in adherence junctions and down-regulated during epithelial to mesenchymal transition (EMT), was significantly down-regulated in the rats that received the lowest dose at PND46. The expression of other markers of the EMT, namely vimentin and N-cadherin, and of the junctional proteins β-catenin, connexin26 and connexin43, were not significantly affected by BFRs treatment. No effects were observed on the estrogen or the progesterone receptors. However, the thyroid hormone receptor alpha (TRα) was significantly down-regulated at puberty (PND46) in the rats that received the lowest dose of the BFR mixture. These results suggest that a perinatal exposure to BFRs can affect protein regulation later on during puberty, a sensitive period of life for mammary development and breast cancer.

Introduction

Unlike most organs, mammary gland development is a multiple stages process, which occurs mainly after birth. At birth, only a rudimentary tree-like ductal structure is present (Hinck et al., 2005). Development resumes at the time of puberty as the ovaries produce a surge of estrogens (Mallepell et al., 2006). A second phase of development occurs during pregnancy, with the development of the milk-secreting structures, the alveoli (Hurley, 1989, Sternlicht et al., 2006). At weaning, the gland goes through an important phase of apoptosis and regression, named involution (Hughes et al., 2012,

Hurley, 1989). Importantly, throughout adult life, the gland undergoes cycles of proliferation-regression with every menstrual cycle (Brisken, 2002).

The human breast contains two major types of tissues: the epithelial tissue, a ramified tree-like structure, and the stroma, mainly composed of extracellular matrices and adipocytes (Hassiotou et al., 2013). Two layers of epithelial cells are present in the former. The luminal cells form the inner layer that encapsulates the ductal lumen while the basal layer, mainly composed of contractile myoepithelial cells, surround the luminal cells and lie on a basal membrane (Hassiotou et al., 2013). A bidirectional cross-talk between these two layers of cells is required for the proper development of the mammary gland, and dysregulation of these interactions has been linked to breast cancer (Bissell et al., 1999, Brisken et al., 2010, Hinck et al., 2005)

Cell-cell interactions involve various mechanisms, including gap junctional intercellular communication (GJIC). Gap junctions (GJ) are specialized transmembrane channels made of a family of proteins named connexins (Cxs). They allow direct communication between adjacent cells (El-Saghir et al., 2011). In rodent mammary gland, Cx26, Cx30 and Cx32 are present between luminal cells in a phase-dependent manner, while Cx43 is mainly expressed between myoepithelial cells at all stages (McLachlan et al., 2007). Down-regulation of GJIC or dysregulation of Cxs has been associated with delayed development and impaired function of mammary gland, as well as increased breast carcinogenesis (Naus et al., 2010). GJ are also closely associated with adherens junctions, which mediate cell-cell adhesion in epithelial cells. Adherens junctions are composed of transmembrane proteins, the cadherins, and cytoplasmic proteins, the catenins (Lien et al., 2006). Down-regulation of E-cadherin is one of the hallmarks of the epithelial to mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal characteristics that enable them to migrate towards surrounding tissues (Eger et al., 2000) EMT causes the epithelial cells to lose their polarity and helps them to gain motility during breast cancer progression (Kalluri et al., 2003).

Since hormones tightly regulate mammary gland development, this organ is especially vulnerable to the effects of endocrine disruptors (EDs). EDs are molecules that mimic or block the effects of endogenous hormones (Rice et al., 2003). Accordingly, a perinatal exposure to various EDs has been associated with developmental defect and increased risk of breast cancer at adulthood (Fenton et al., 2012). Similarly, in human, epidemiological studies have demonstrated that exposure to EDs increases the risk for breast cancer (Jenkins et al., 2012). The odds of developing breast cancer are even more

important if women are exposed during vulnerable periods involving important remodelling of the mammary glands, such as pregnancy, lactation or early life (Fenton et al., 2012). Brominated flame retardants (BFRs) are chemicals widely used in consumer products to reduce flame propagation and ignition rates (Berger et al., 2014). They can be found in electronics, vehicles, plastics and textiles, amongst others. Evidence suggests that BFRs act as EDs, having anti-androgenic and estrogenic properties (Y. R. Kim et al., 2014). Also, studies have shown that BFRs may interfere with thyroid hormone regulation (Y. R. Kim et al., 2014). However, the role of BFRs in mammary gland development and breast cancer, as well as the mechanisms implicated in the compound's toxicity, are poorly understood.

This study aimed to evaluate the impact of BFRs exposure on mammary gland. More specifically, the goals were to 1) assess the effects of perinatal exposure to BFRs on mammary gland development and breast cancer in the female offspring, and 2) determine mechanisms implicated in BFRs toxicology.

Materials and methods

Animals

Animals and treatment methods were described previously (Lefevre et al., 2016). Briefly, virgin female Sprague-Dawley rats were obtained from Charles River Laboratories. Animals were housed individually at Health Canada in rooms maintained at 20°C on a 12L: 12D photoperiod. Food and water were provided ad libitum. All animal studies were conducted in accordance with the procedures and principles outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care under protocol 4456. Following one week of acclimatization to the control diet, female rats were randomly assigned to one of four experimental conditions (N = 35-38 per group) and fed a BFR supplemented diet for 2-4 weeks before mating. During this period, estrous cyclicity was evaluated by analyzing vaginal cytology as previously described (Goldman et al., 2007). Females in proestrus were caged with proven breeder male Sprague-Dawley rats (maintained on the control diet) overnight.

Sample collection

Pups were euthanized with CO2 immediately followed by heart puncture at post-natal day 21 (PND21), PND46 and PND208. Mammary glands #4 and #5 (abdominal and inguinal, respectively) were removed

and weighed. Both left and right inguinal mammary glands as well as left abdominal mammary glands were snap frozen immediately after excision and stored at -80°C. Right abdominal mammary glands were immediately transferred onto large slides then fixed in Carnoy and stained. Mammary glands #2 and #3 (upper-thoracic and thoracic, respectively) were excised (right side only). They were embedded in tissue-tek O.C.T compound (VWR) on dry ice and stored at -80°C.

Brominated Flame Retardants Formulation

Formulation of the BFR mixture was described previously (Ernest et al., 2012). Briefly, three technical PBDE mixtures (DE-71, DE-79 and BDE 209) and one HBCD mixture were combined to yield a ratio of PBDE congeners and HBCD comparable to the median levels observed in Boston house dust (Allen et al., 2008, Stapleton et al., 2008). This BFR mixture was incorporated into an isoflavone-free diet (Teklad Global 2019 diet; Harlan Laboratories, Madison, WI) with 4.3g/kg corn oil. Diets were formulated to contain 0, 0.75, 250 or 750 mg of BFR mixture/kg. The diet formulations were intended to deliver nominal doses of 0, 0.06, 20 and 60mg/kg body weight/day. The lowest dose was estimated to be a close approximation of maximum human exposure, based on a dust ingestion rate of 100mg/day in children (16.5kg body weight) and the scaling of dose from humans to rodents (1:6.9, human to rat body surface area ratio) (Allen et al., 2008, Stapleton et al., 2008).

Masson Trichromatic Staining

Sections of 10um of thickness were obtained by cryosection at -35°C from mammary glands embedded in tissue-tek O.C.T compound (VWR) and transferred onto slides. Slides were fixed in Bouin's solution overnight and rinsed with tap water (10 minutes). Slides were incubated in Weigert's iron hematoxylin (10 minutes), in Biebrich scarlet-acid fuchsin (15 minutes), in phosphomolybdic-phosphotungstic acid (20 minutes), submerged in aniline blue solution (5 minutes) and in 1% acetic acid (5 minutes). Slides were dehydrated in 95% ethyl alcohol (5 minutes), absolute ethyl alchohol (5 minutes) and xylene (5 minutes). Slides were mounted with resinous mounting medium (permount).

Ductal elongation and surface area measurements

Ductal elongation and surface area were measured using pictures of mammary gland whole mounts and Image J software. Ductal elongation was measured by tracing a line from the proximal lymph node to the end of the longest branch. Surface area was measured by tracing the best-adjusted contour of the mammary epithelium.

Protein extraction

500 mg sections of snap-frozen mammary gland were cut and mechanically transformed into a powder on dry ice. Powdered samples, once weighed, were snap frozen in liquid nitrogen. Triple detergent lysis buffer (pH: 8) (Tris 50 mM, NaCl 150 mM, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate) that was supplemented with NaF 1.25 M, NaVO3 1 M and Halt Protease and Phosphatase Cocktail Inhibitor (Fisher Scientific) was added to each sample. Each sample, dissolved in lysis buffer, was sonicated after 30 minute incubation at 4°C. After a 20 minute centrifugation at 4°C, supernatants were aliquoted and stored at -80°C until further processing. All steps were performed on ice.

Western Blot

Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo scientific #23227). For each sample, 30 µg of protein was separated onto SDS-PAGE gels (TGX Stain-Free FastCast Acrylamide kit, 10%, BioRad, Mississauga, ON) and transferred onto PVDF membranes. Membranes were blocked with TBS-Tween 0.1 % Tween 20 containing 3 % bovine serum albumin (BSA) and incubated overnight at 4°C with primary antibodies (B-catenin (1/1000), Cell Signaling (8480); E-cadherin (1/1000), Cell signaling (14472); Cx43 (1/5000), Sigma Aldrich (C6219); Cx26 (1/750), Invitrogen (33-5800); ERα (1/400), Abcam (Ab75635); ERβ (1/500) Santa Cruz (SC-8974); PR-A/B (1/500), Abcam (ab16661); STAT1 (1/1000), Cell signaling (9172); N-cadherin (1/1000), Cell signaling (4061); Vimentin (1/1000), Cell signaling (5741); Thyroid Hormone Receptor α (TRα) (1:500), Abcam (Ab53729); TRβ (1/1000), Abcam (Ab196484)). Horseradish peroxidase (HRP)-conjugated secondary antibodies (goat-anti-rabbit (1/10 000), Cell signaling (7074); horse-anti-mouse (1/10 000), Cell signaling (7076)) were used. Chemiluminescent signals were detected using the Clarity western ECL substrate (BioRad) and analysed using ChemiDoc MP System and ImageLab software (BioRad). Protein expressions were normalised on total lane protein.

Statistic

Data were analyzed using GraphPad (6th edition) using a Student t test or ANOVA analysis followed by Tukey's multiple comparison test when conditions of applications were respected. Otherwise, a Kruskal-Wallis test followed by a Dunn's multiple comparison test were used.

Results

BFRs exposure has no significant effect on global mammary gland development

In order to assess global toxicity of the BFRs mixture, we first measured total body weights and mammary gland weights at time of sacrifice (Figure 1). At all time points, treatment with BFRs did not significantly alter body weights. Similarly, mammary gland weights were not affected by the treatments (Figure 1B, D, F).

Next, to assess the impact of the BFRs treatment on mammary gland morphogenesis, we further assessed mammary gland development by measuring the elongation and surface area of the epithelium. Consistent with normal development of the mammary gland, at PND21, ducts and branches from control mice were elongated away from the lymph node, and terminal end buds (TEBs) were numerous (Figure 2A, B). At PND46, ducts reached toward the periphery of the fat pad and side branches were abundant (Figure 2C, D).

Finally, at PND208, the ductal system was well implanted in the fat pad with numerous side branches with little or no evidence of TEBs (Figure 2E, F). Whole mounts showed similar epithelial growth for the control and the treated groups (Figure 2). TEBs were also counted at PND46, the time of puberty, to assess normal development but also normal timing of development. Once again, this marker was not significantly affected by perinatal exposure to the BFRs mixture (Figure 2G).

We then examined the effect of BFRs on mammary gland structure using Masson Trichrome staining on mammary gland tissue sections. As expected, the sections of mammary gland at PND46 showed a few ducts, surrounded by many adipocytes (Figure 3). The luminal cells delimit the lumen and the myoepithelial cell layer is surrounded by extracellular matrix that contains a lot of collagen and fibroblasts (Figure 3). Lymph nodes are also observed encompassed in extracellular matrix (Figure 3). No significant differences were observed in the treated groups when compared to control. Together, our results showed that a perinatal exposure to a mixture of BFRs did not affect global mammary gland development. However, some developmental markers could still be affected by the treatment.

BFRs-induced E-cadherin down-regulation is not associated with modulation of other markers of the epithelial to mesenchymal transition (EMT)

After ruling out large-scale anatomical effects with whole mount techniques, we proceeded with western blot in order to identify changes in protein expression. Epithelial to mesenchymal transition (EMT) is a hallmark of cancer. Because it has been suggested that a perinatal exposure to EDs is linked to breast cancer later on in adulthood (Fenton et al., 2012), we first wanted to determine if BFRs could promote EMT in rat mammary gland. Cells undergoing this transition can be identified by their downregulation of epithelial markers such as E-cadherin and their upregulation of mesenchymal markers such as N-cadherin and Vimentin. Our results showed that E-cadherin expression was down-regulated at puberty (PND46) in the group that received the lowest dose of the BFRs mixture (Figure 4B), but not at PND21 and PND208 (Figure 4A, B).

We next evaluated the expression of two mesenchymal markers, namely Vimentin (Figure 5A) and N-cadherin (Figure 5B) by western blot in the PND46 rats. Our results showed that while the epithelial marker E-cadherin was significantly down-regulated in the group that received the lowest dose (Figure 4A), neither of the mesenchymal markers were up-regulated in treatment groups when compared to the control group (Figure 5), indicating that EMT is not occurring in response to BFRs treatment.

BFR treatment has an impact on adherence junction, but not on gap junctions

Because E-cadherin was decreased but other markers of EMT were not modified by the treatment (Figures 4, 5), we then speculated that the cell-cell interactions nexus rather that EMT was affected by the treatment. We thus determined the effect of BFRs on β-catenin, and Cx43 and Cx26, components of adherens junctions and GJ, respectively, which changes in expression has been linked to breast cancer and developmental defects. We showed that while Cx43 protein expression seems to be down-regulated in rats that received the lowest dose of the BFR mixture, Cx43 and Cx26 protein expression were not significantly affected by BFR treatment (Figure 6A, B, E, F). Similarly, we found that β-catenin was not significantly affected by the treatment (Figure 6 C, G)

STAT1, a developmental marker, is unaffected by treatment with the BFRs mixture at PND46

STAT1 is known to be present in mammary gland in virgin rodents. While its function during mammary gland development is poorly understood, dysregulation of STAT1 has been linked to tumor suppression in breast cancer (Koromilas et al., 2013). We thus next assessed STAT1 levels. The protein expression of STAT1 was not significantly altered by treatment with BFRs (Figure 7A). STAT1 is a transcription factor that undergoes dimerization and nuclear translocation when phosphorylated. Phospho-STAT1 could

not be detected in both control and treated animals (Figure 7B), suggesting that STAT1 was not activated in our tissue samples.

Thyroid Hormone Receptor alpha (TRα) is down-regulated by BFRs treatment at puberty whereas Estrogen and Progesterone receptors are not affected

While intercellular junctions are known to play a role in mammary gland development, hormones and their receptors are essential to normal development, especially during puberty. At this time point, the two most important hormones are estrogen and progesterone and their receptors, ERα and PR-B, ensure proper signalling. Since BFRs are known EDs, it was crucial to verify whether the treatment affects hormone receptor's expression. ERα (Figure 8A, E) and PR-A/B receptors (Figure 8B, F), as well as ERβ (data not shown), were not affected by perinatal BFR exposure.

In previous studies with BFRs, the systemic level of thyroid hormones was significantly reduced by BFR treatment. While thyroid hormones are not essential for mammary gland development, dysregulation of their receptors has been linked to breast cancer (Conde et al., 2006). For these reasons, we investigated the thyroid hormone receptors present in the mammary gland: TRα and TRβ. TRα protein expression was significantly down-regulated in rats that received the lowest dose of the BFRs mixture (Figure 8C, G). While TRβ protein expression seems to follow the same pattern, these changes were not statistically significant (Figure 8D, H).

Discussion

BFRs are chemicals widely used in consumer products to reduce ignition rates. Although uses of particular congeners have been restricted few years ago, their concentrations remain high in various human tissues and matrices, including milk and mammary gland. Studies have demonstrated that BFRs have endocrine disruptive properties. Thus, this study aimed to determine whether a perinatal exposure to BFRs could affect mammary gland development or promote breast cancer. Our results showed that gross mammary gland development was not affected by BFRs. Similarly, markers of EMT and components of the gap junctions studied were not affected at puberty. However, the treatment down-regulated E-cadherin and TRα expression at a dose which is considered a close approximation to human exposure. Together, our results showed that while little effects were observed, a perinatal exposure to BFRs at an environmentally relevant dose could affect protein expression later on, at puberty.

Mammary gland development is not affected by a perinatal exposure to BFRs

Very few studies have considered the impact of BFRs exposure on mammary gland development. One study, conducted with rats, sought to determine the effects of a perinatal exposure to a commercial PBDE mixture, DE-71, on neurobehavioral, hormonal and reproductive outcomes (Kodavanti et al., 2010). The authors found that there was a significant delay in mammary gland development in the female offsprings of rats treated during pregnancy and lactation. Outgrowth of the epithelium was suppressed at post-natal day 21 (PND21) as assessed by carmine staining and subsequent scoring. There was also fewer TEBs and branches at the time of puberty (Kodavanti et al., 2010). Our results, however, suggest that gross epithelial growth was not affected by perinatal exposure to aBFRs mixture. This discrepancy could be due to the difference in the composition of the BFRs mixture used. DE-71 is a widely used Penta-PBDE while our mixture was composed of three technical PBDE (DE-71, DE-79 and BDE 209) and one HBCD, combined to yield a ratio of PBDE congeners and HBCD comparable to the median levels observed in Boston house dust (Allen et al., 2008, Stapleton et al., 2008). While using DE-71 alone can inform us on the direct impact of this mixture, the BFRs mixture used in this study allows us to better characterize the impact of BFRs in terms of current exposures.

Another possible explanation for this discrepancy is the different strains of rats that were used. Studies have shown that mammary tumour formation following Atrazine exposure was strain dependant as Sprague-Dawley and F344 rats responded differently (Eldridge, 1998). Others have compared mammary gland development following Atrazine prenatal exposure in Sprague-Dawley and Long-Evans rats and have identified significant differences (Wolf, 2010). This may explain why Kodavanti et al (2010) observed significant delay in mammary gland development in Long-Evans rats exposed to BFRs while we did not observe these effects in our Sprague-Dawley rats.

A perinatal exposure to BFRs had no effect on chosen markers of EMT or gap junctions

Few studies have assessed the impact of BFRs on breast cancer development or progression and the limited data available is not very conclusive. One in vitro study has shown that PBDE-209 stimulates breast cancer cell proliferation in the MCF-7 cell line (Li et al., 2012). Another study with MCF-7 cells showed PBDE were capable of damaging cell genome (Barber et al., 2006). However, a case-control study, that assessed the link between adipose tissue concentrations of PBDEs and breast cancer risk in California, found no such association (Hurley, 1989).

It has been shown, in lung cancer cells as well as liver carcinoma cells, that BFRs can promote cancer cell migration and invasion through the activation of EMT (Qu et al., 2015, Zhong Y, 2014). More specifically, 6-OH-BDE-47 induction of EMT in lung cancer cells was confirmed by down regulation of epithelial markers (E-cadherin and Zona Occludin-1 (ZO-1)) as well as up regulation of mesenchymal markers (Vimentin and N-cadherin) (Qu et al., 2015). E-cadherin down-regulation was also detected in human mammary stem cells in response to BPA, another endocrine disrupting chemical that has been studied more extensively (Yang et al., 2013). Based on these results, we assessed the effects of our BFRs mixture on various EMT markers in mammary glands. We found that E-cadherin was significantly down-regulated at the time of puberty in the group that received the lowest dose. Despite that, the other epithelial and mesenchymal markers were unaffected which suggests that EMT was not likely activated in our tissues. However, we assessed a very limited amount of markers: E-cadherin, β-catenin, Vimentin and N-cadherin. In order to confidently rule out the activation of EMT, it would be necessary to assess different EMT markers such as snail or slug, two transcription factors that are upregulated during EMT (Kokudo et al., 2008, Medici et al., 2008).

E-cadherin is a major component of adherens junctions. Many studies have demonstrated that components of adherens junctions interact, co-localize and immunoprecipitate with components of GJ (Talhouk et al., 2013, Talhouk et al., 2008, Wu et al., 2003, Xu et al., 2001) Moreover, E-cadherin and Cxs down-regulate concomitantly in many tumors, and can be regulated through common pathways (Plante et al., 2006, Plante et al., 2005). This is why a down-regulation in E-cadherin protein expression led us to believe that BFRs exposure might affect GJ proteins Cx26 and Cx43. Very limited data exists on the impact of BFRs exposure on GJ. Tetrabromobisphenol A (TBBPA), one of the main BFRs, has been shown to inhibit GJIC in an epithelial liver cell line (Samuelsen, 2014). In the present study, we found that Cx26 was unaffected by a perinatal exposure to BFRs. While there seems to be a trend for downregulation of Cx43 expression in rats that received the lowest dose, this effect was not statistically significant. Nevertheless, it would be important to study protein localization as Cxs can only participate in GJIC when they are located at the plasma membrane (Xie et al., 1997).

ER and PR are not affected by a perinatal exposure to BFRs

It has been demonstrated that BFRs can have estrogenic properties and affect progesterone signalling (Ceccatelli et al., 2006). However, to our knowledge, this is the first study to assess the effect of BFRs exposure on mammary ER and PR expression. One in vitro study has described the PR antagonistic

activity of different BFRs, including PBDE and HBCD (Hamers et al., 2006). A few studies have described an interaction between BFRs and estrogen signalling (Ceccatelli et al., 2006, Hamers et al., 2006, Meerts et al., 2001). Meerts et al (2001) used luciferase assays to determine that the higher-brominated PBDE had an antagonistic effect on ER while the lower brominated PBDE had an agonistic effect. These findings were later corroborated by Hamers et al (2006) during an in vitro study with other PBDE congeners. Moreover, it has been suggested that BFRs can exert their estrogenic effect by increasing the bioavailability of endogenous estrogens (Kester et al., 2002). The inhibition of estradiol sulfotransferase enzymes led to decreased sulfation and excretion of estradiol (Kester et al., 2002). Similarly, rats injected with PBDE99 showed dysregulation of estrogen target genes in uterus, including a dowregulation in PR levels, while ERα and ERβ uterine mRNA levels were increased (Ceccatelli et al., 2006). On the opposite, our results indicate that BFRs do not affect ERα or PR protein expression in the mammary gland. This discrepancy between our results and the ones obtained by Ceccatelli et al (2006) could be explained by the differences in tissues (uterus versus mammary gland) or markers (protein versus mRNA) used. It could also be explained by the method of exposure (injection versus dietary intake) or by the specific BFRs used. As previously described by Meerts et al (2001), some congeners have an antagonistic effect while others have an agonistic effect. Thus, although the estrogenic effect is lost using a mixture of BFRs, it is more representative of human exposure.

TRα is down-regulated at puberty by a perinatal exposure to BFRs

Many studies have demonstrated an effect of BFRs on thyroid hormone homeostasis including decreases in thyroxine (T4) levels and increases in thyroid stimulating hormone (TSH) levels in both exposed pregnant rats and their female offsprings (Ernest et al., 2012, T. H. Kim et al., 2009, Kodavanti et al., 2010, van der Ven et al., 2006). Decreased serum T4 levels could be linked to a competition between some BFRs and T4 for binding to human transthyretin, the T4-transporting protein in plasma, as demonstrated in vitro (Hamers et al., 2006, Meerts et al., 2000).

The effect of BFRs on TRs signaling has been studied. The association between PBDE and TRβ, was assessed in vitro. No direct interaction between the two could be identified. Also, the PBDE did not compete with T3 for binding to the TR (Suvorov et al., 2011). This same in vitro study failed to find a role for BDE-47 in the regulation of TRβ transcriptional activity (Suvorov et al., 2011). However, they reported a differential expression of thyroid responsive genes in liver and brain following a BDE-47 exposure (Suvorov et al., 2011). Interestingly, it has been shown that T3 responsive genes display a

marked preference for one of the two receptors (TRα or TRβ) and that these two receptors can exert different functions (Chatonnet et al., 2013). This could explain the differential expression of thyroid responsive genes in the liver and the brain despite the lack of in vitro effect on TRβ transcriptional activity.

To the best of our knowledge, the effect of BFRs on the TR protein expression in mammary gland has never been assessed. Our results show a significant downregulation of TRα, but not TRβ, at the time of puberty in female offsprings exposed perinatally to the BFRs mixture. Thyroid hormones are involved in mammary gland development and differentiation. Specifically, they have been shown to regulate tertiary epithelial branching and lobulo-alveolar development in mice (Hovey et al., 2002). They also influence mammary development through their activation of growth hormone (GH) secretion (Meites et al., 1964) and through their upregulation of epidermal growth factor receptor (EGFR) (Vonderhaar et al., 1986). Given these roles for TRs in mammary development, we would expect a downregulation in TR protein expression to restrict breast development. In breast cancer, the expression and localization of TRs are altered, which suggests that a dysregulation at this level might promote mammary tumor development (Conde et al., 2006).

A perinatal exposure to an environmental dose of BFRs alters protein expression at puberty

We observed protein expression dysregulation uniquely in the group that received the lowest dose. Interestingly, this dose is the one that best represents human exposure based on house dust levels and dust ingestion rates (Allen et al., 2008, Stapleton et al., 2008). Furthermore, our results suggest that perinatal exposure affects mammary glands later on at the time of puberty. Modifications, such as altered hormone signalling, at the time of puberty have been shown to increase the risk of breast cancer development (Boylan et al., 1983). In this study, the timing of the observed effects could have important long-term consequences, as puberty is known to be a sensitive period for the development of breast cancer. To our knowledge, no association between E-cadherin and TR protein expression have been reported in literature, which indicates that BFRs could potentially affect mammary gland by two completely different mechanisms. Further analyses are needed to determine the mechanisms involved in TRα and E-cadherin down-regulation, and whether or not both are linked.

Conclusion

Overall, our results showed that while no effect were observed on global mammary gland development, expression of E-cadherin, a protein involved in adherens junctions and involved in breast cancer, and TRα are down-regulated in the rats that received the lowest dose at PND46. Thus, our results suggest that exposure to an environmentally relevant BFRs mixture can affect the endocrine system as well as cell-cell adhesion in the mammary gland during puberty. Importantly, our results showed that a perinatal exposure to BFRs could affect protein regulation later on during puberty, a sensitive period for mammary development and breast cancer. Whether or not other markers are affected and the long-term effects of those down-regulations remain to be determined.

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Figure legends

Figure 1. A perinatal exposure to a BFR mixture did not affect body and mammary gland weights. Female rats were exposed to BFRs perinatally and sampled at PND21 (A-B) PND46 (C-D) and PND208 (E-F) Body weights (A, C, E) and mammary gland weights were measured at time of sacrifice. Mammary gland weights were normalized to total body weights (B, D, F). Graphs represent mean ± SEM (N = 9-13).

Figure 2. Mammary gland morphogenesis is not affected by a perinatal exposure to BFRs. Female rats were exposed to BFR perinatally and sampled at PND21 (A-B), PND46 (C-D, G) and PND208 (E-F). Whole mounts of mammary glands were analyzed with Image J software. Elongation was measured from the lowest lymph node to the end of the longest branch of the epithelium (A, C, E). Surface area was measured (B, D, F) and terminal end buds were counted on whole mount images (G). Graphs represent mean ± SEM (N =7-13).

Figure 3. Perinatal exposure to BFR mixture did not alter mammary gland structure. Female rats were exposed to BFR perinatally and mammary glands were sampled at PND46. Sections of 10um of thickness were obtained by cryosection at -35°C and stained with Masson Trichrome Staining. Mammary epithelium from control as well as low, medium and high doses treatment groups are shown at a magnification of 40X. The luminal cells delimit the lumen and the myoepithelial cell layer (in red) is surrounded by extracellular matrix that contains a lot of collagen (in blue) and fibroblasts (in blue with red nuclei). Lymph nodes are also observed (in pink) encompassed in extracellular matrix (in blue). Images show epithelial ducts (yellow arrow), adipocytes (black arrow), fibroblasts (white arrow), collagen (white arrowhead), blood vessels (red arrow) and lymph nodes (green arrow).

Figure 4. E-cadherin protein expression is only affected during puberty. Total cell lysates from control group (C) and low (L), medium (M) and high (H) dose treatment groups were analyzed by western blot (A-C). Protein expression for E-cadherin was measured at PND21 (A), PND46 (B) and PND208 (C). Total lane protein was used for normalization. Graphs (D-F) represent mean ± SEM (N = 9-13).

Figure 5. N-cadherin and Vimentin are not affected by a BFRs treatment at PND46. Total cell lysates from control group (C) and low (L), medium (M) and high (H) dose treatment groups were analyzed by western blot (A-B). Protein expression for N-cadherin (A, C) and Vimentin (B, D)) was measured. Total lane protein was used for normalization. Graphs (D-F) represent mean ± SEM (N= 9-13).

Figure 6. BFR treatment has no impact on gap junctions. Total cell lysates from control group (C) and low (L), medium (M) and high (H) dose treatment groups were analyzed by western blot (A-D). Protein expression for Cx43 (A), Cx26 (B) and β -catenin (C) was measured. Total lane protein was used for normalization. Graphs (D-F) represent mean ± SEM (N= 9-13).

Figure 7. STAT1 is unaffected by treatment with the BFR mixture at PND46. Total cell lysates from control group (C) and low (L), medium (M) and high (H) dose treatment groups were analyzed by western blot (A, B). Protein expression for STAT1 (A) and phospo-STAT1 (B) was assessed. Total lane protein was used for normalization. Graph (C) represent mean ± SEM (N = 9-13).

Figure 8. Thyroid Hormone Receptor alpha (TRα) is down-regulated by BFRs treatment whereas Estrogen and Progesterone receptors are not. Total cell lysates from control group (C) and low (L), medium (M) and high (H) dose treatment groups were analyzed by western blot (A-D). Protein expression for estrogen receptor α (A), progesterone receptor A and B (B), thyroid hormone receptor α

(C) and β (D) was measured. Total lane protein was used for normalization. Graphs (E-H) represent mean ± SEM (N=9-13).

Figures

Figure 1

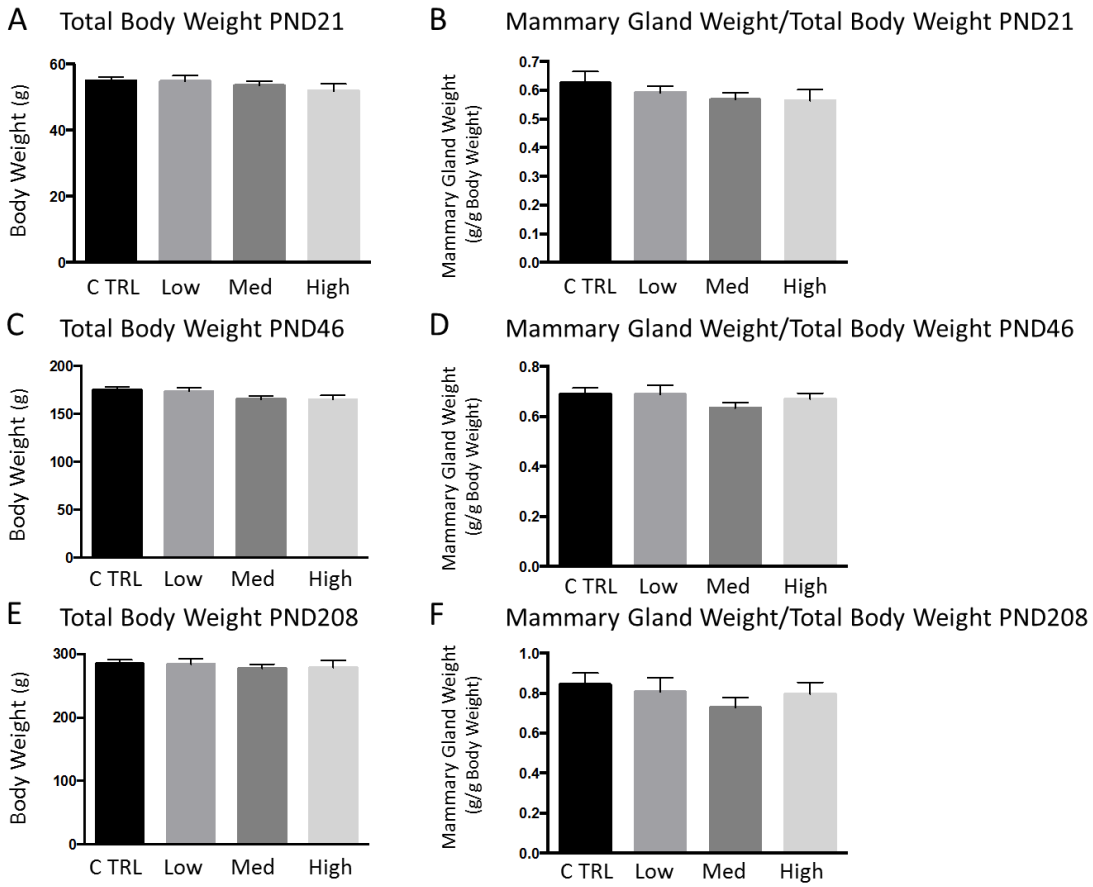


Figure 2

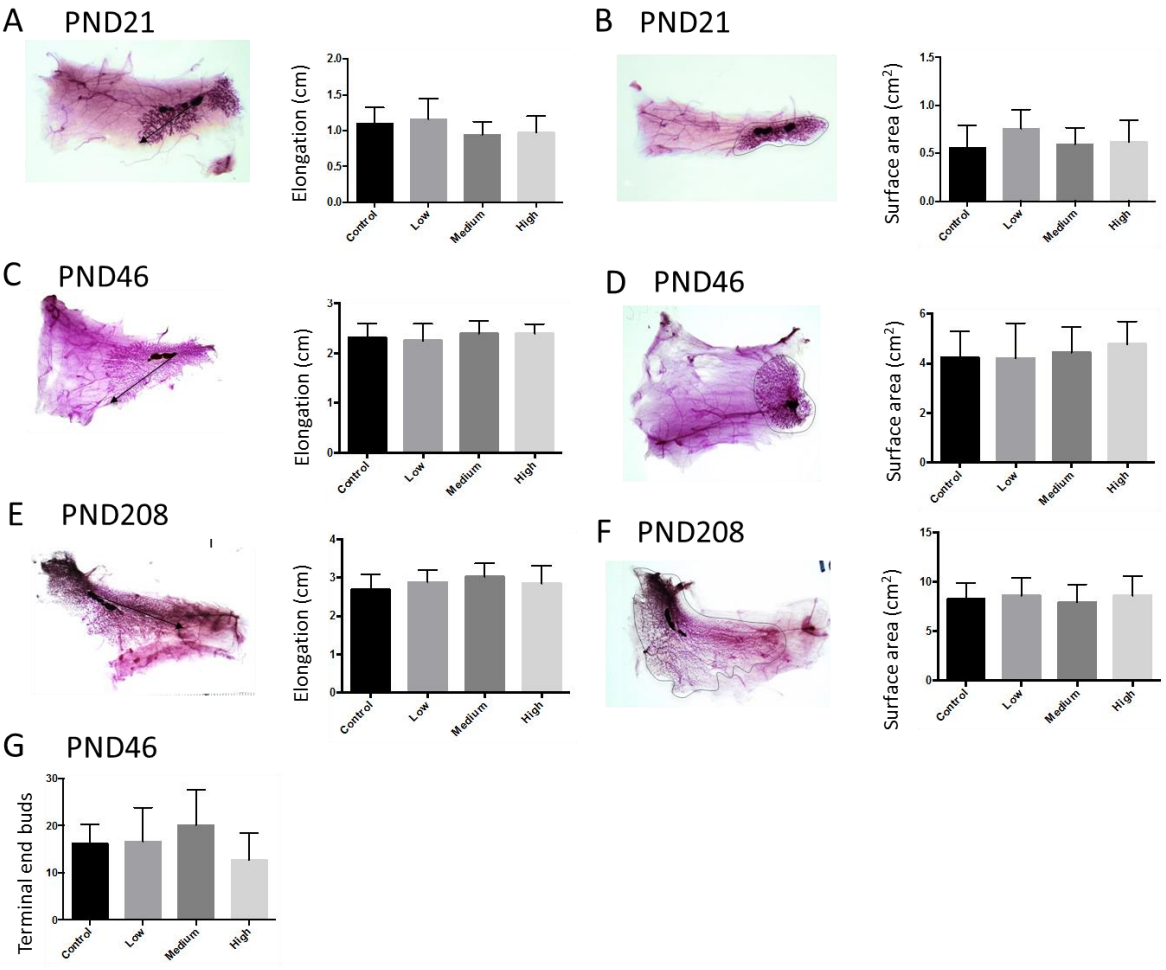
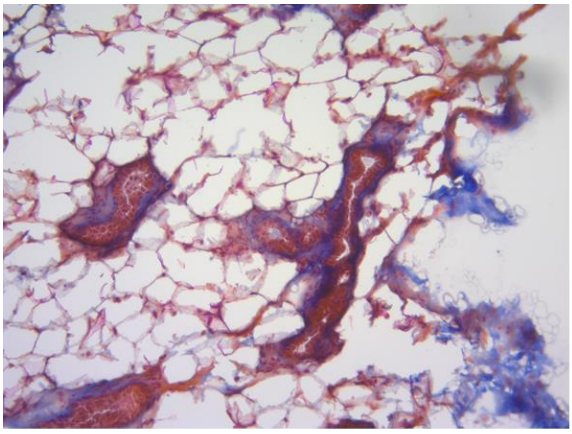

A PND21
Elongation (cm)
Control
Low
Medium
High
B PND21
Surface area (cm2)
C PND46
D PND46
E PND208
F PND208
G PND46
Terminal end buds

Figure 3



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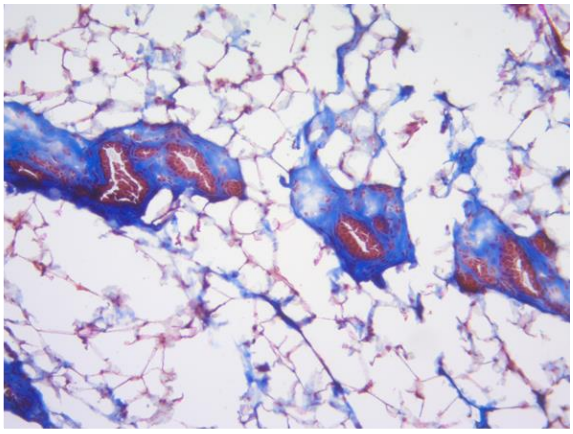

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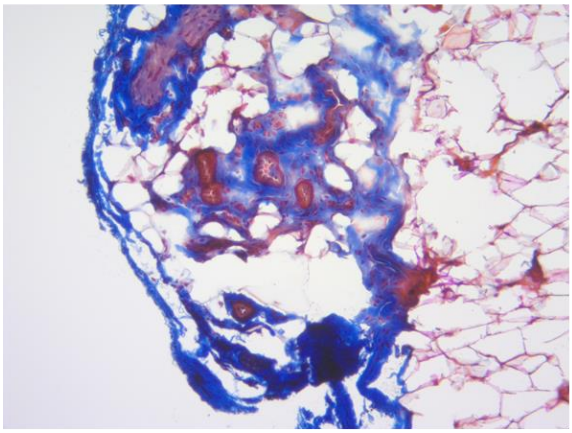

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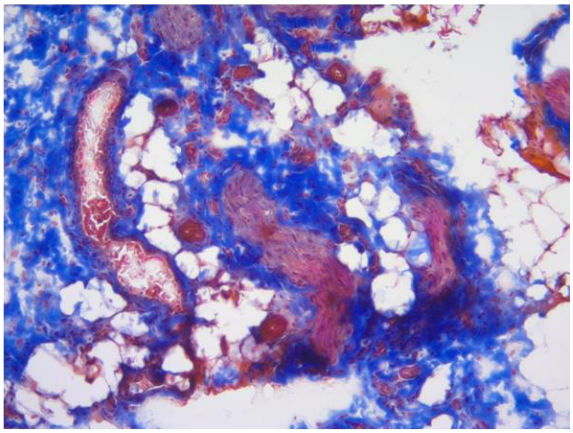


High

Figure 4

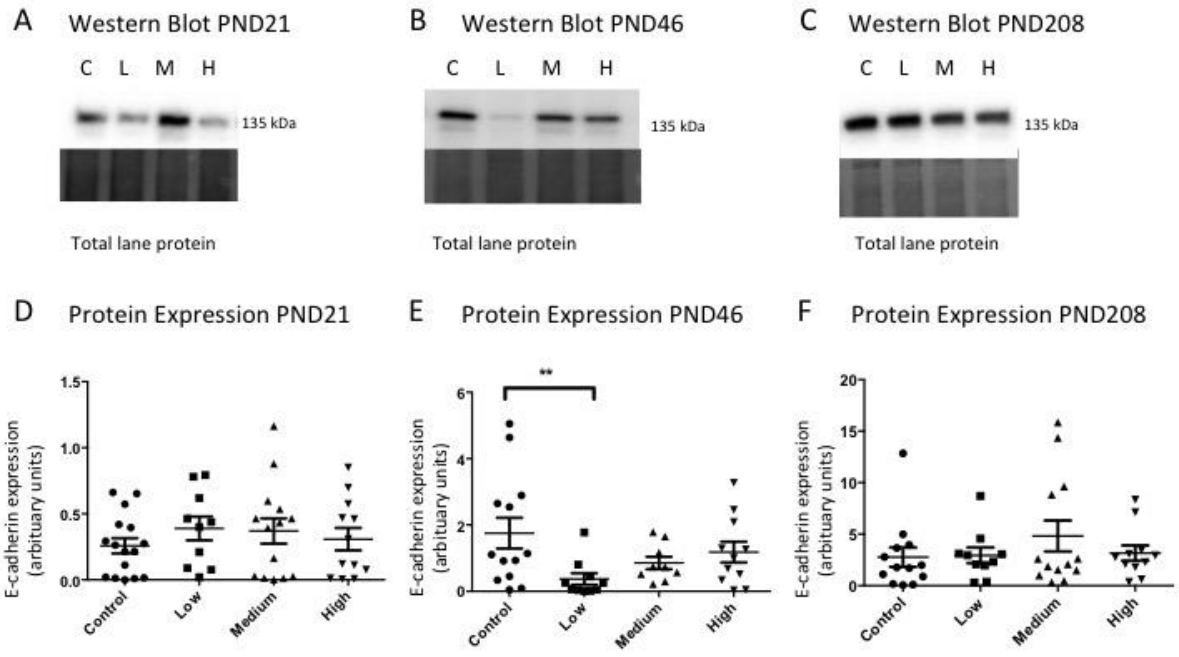


Figure 5

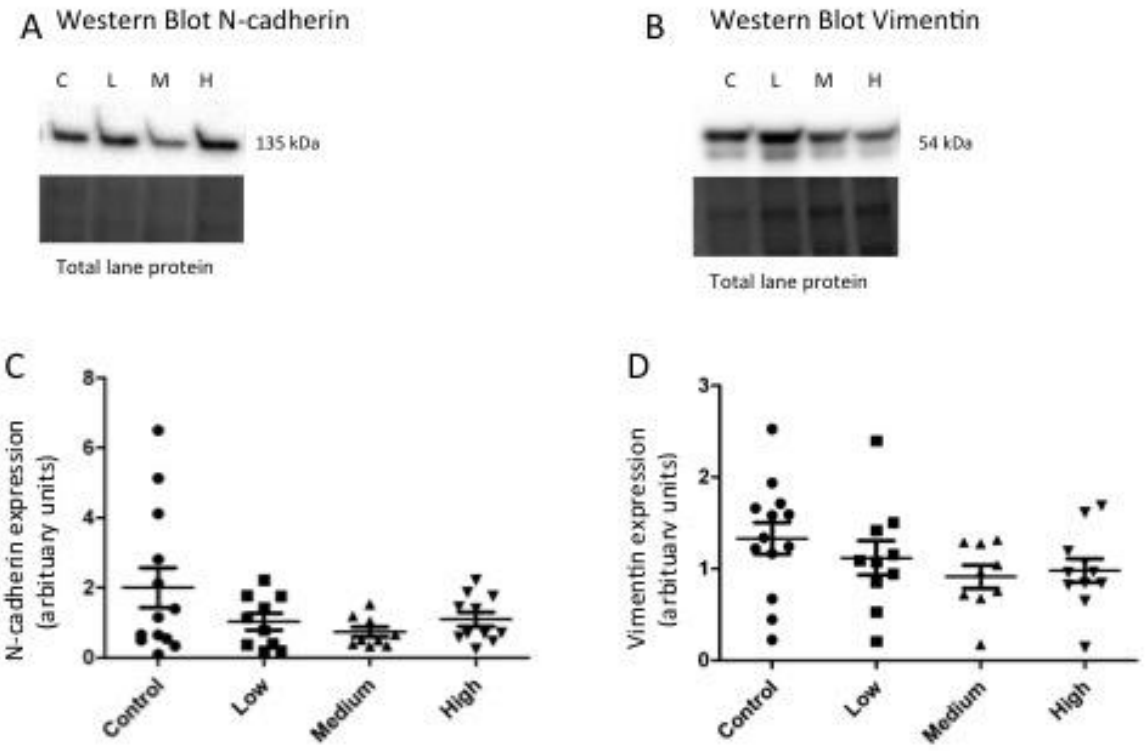


Figure 6

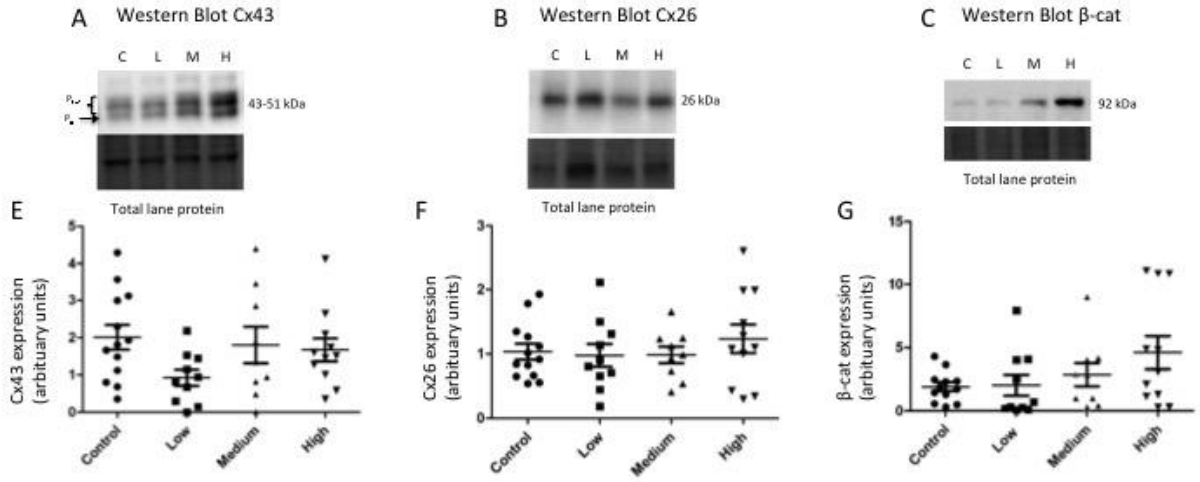


Figure 7

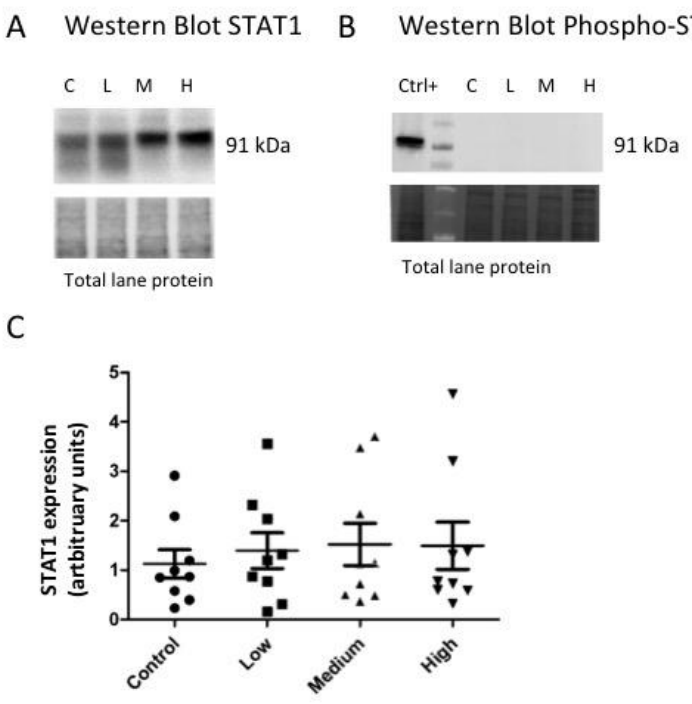
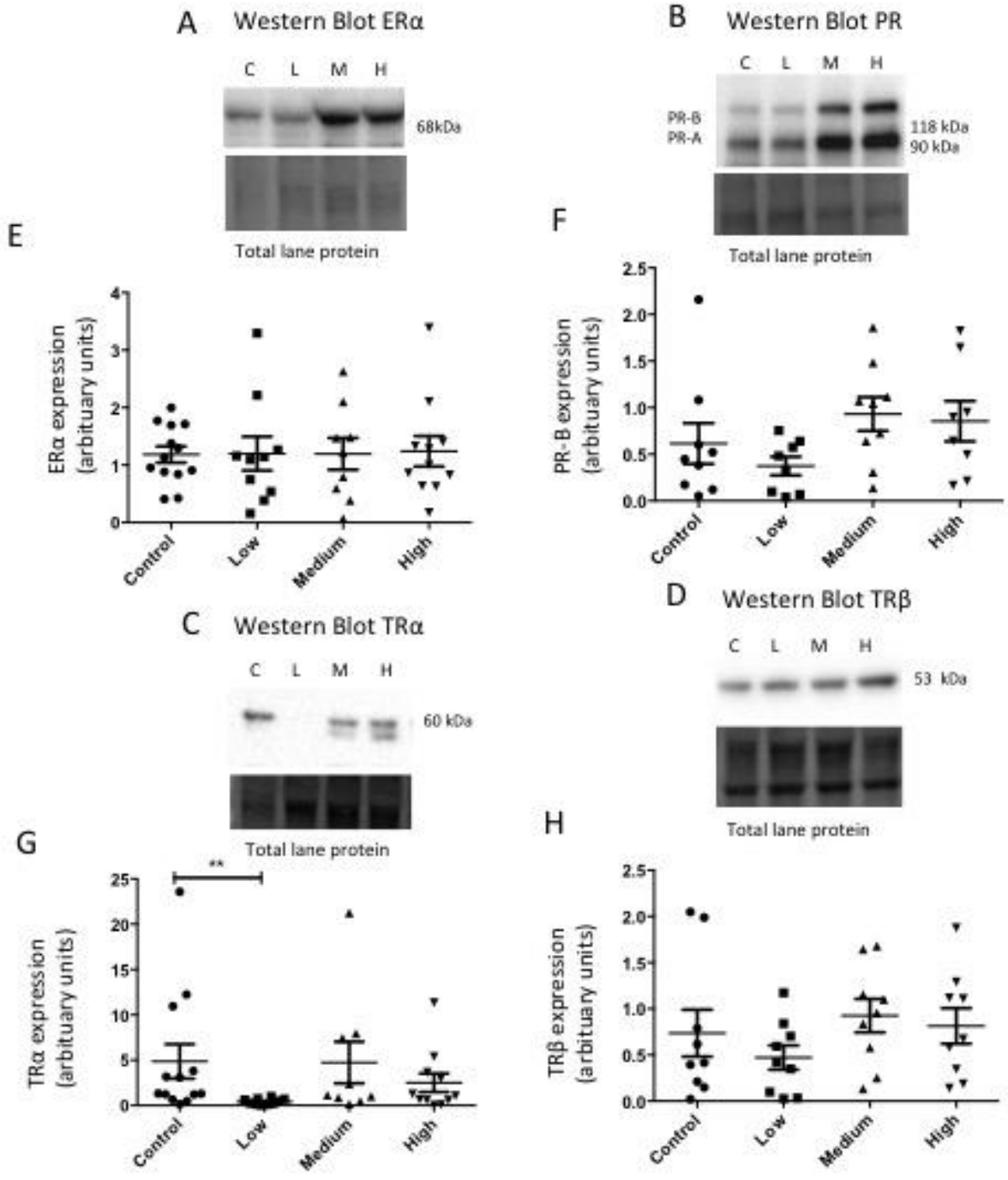

A
Western Blot STAT1
C L M H
91 kDa
Total lane protein
B
Western Blot Phospho-STAT1
Ctrl+ C L M H
91 kDa
Total lane protein
C
STAT1 expression (artbitruary units)
0
1
2
3
4
5
Control
Low
Medium
High

Figure 8


A
Western Blot ERα
C L M H
68kDa
Total lane protein
B
Western Blot PR
C L M H
PR-B
PR-A
118 kDa
90 kDa
Total lane protein
E
ERα expression (arbituary units)
Control
Low
Medium
High
F
PR-B expression (arbituary units)
Control
Low
Medium
High
C
Western Blot TRα
C L M H
60 kDa
Total lane protein
D
Western Blot TRβ
C L M H
53 kDa
Total lane protein
G
**
TRα expression (arbituary units)
Control
Low
Medium
High
H
TRβ expression (arbituary units)
Control
Low
Medium
High

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