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THE ROLE OF MICRORNA IN MAMMARY GLAND DEVELOPMENT AND THE REGULATION OF CONNEXIN 43

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ABSTRACT

Connexins are indispensable to mammary gland development by vitue of allowing gap junction intercellular communication within the luminal and myoepithelial layers of the mammary gland epithelium. It is well-documented that the expression level of connexins is specific to the stage of mammary gland development; however, their mechanisms of regulation, especially by microRNA (miRNA), remains poorly characterized.

The hypothesis of this project was that miRNA vary in a stage-specific manner throughout mammary gland development, and are regulated by key hormones directing its morphogenesis. It was also hypothesized that such regulation is linked with connexins, in particular Cx43, and that hormones and miRNA functionally regulate its mRNA. With this, our objectives were to measure miRNA expression at various stages of development *in vivo*, assess the effect of hormones on both miRNA and Cx43 mRNA *in vitro*, and confirm the regulation of Cx43 mRNA by miRNA by altering their levels *in vitro*. To do so, we extracted and sequenced miRNA from a) mouse mammary glands at four important stages of development, and b) luminal breast cancer cells exposed to estradiol, progesterone, prolactin and oxytocin. Those same hormones were also used in short-term exposures, to determine their effect on Cx43 mRNA.

It was found that miRNA vary substantially as individuals throughout development, but also as clusters that shed light on their broad expression patterns. Based on their expression and on the effects of hormones, miRNA likely involved in mammary gland development in a stage-specific manner were identified. *In vitro*, estradiol and progesterone increased Cx43 mRNA, and oxytocin decreased Cx43 mRNA in short bursts. In addition, prelimiary prolactin experiments indicate that this hormone increases Cx43 mRNA shortly after exposure, but ends up decreasing Cx43 mRNA. Those same hormones resulted in miRNA identified as associated with estradiol, progesterone, prolactin and oxytocin exposure.

Interestingly, both miRNA and connexins are dysregulated during breast cancer in a way that favours the tumour microenvironment, promoting progression and metastasis. The project will thus add important insights on mammary gland development, but is also a prelude to further studies on one of the most serious diseases affecting women worldwide.

Key words: Connexin 43 (Cx43), microRNA (miRNA), hormones, gap junction, intercellular communication, cancer

RÉSUMÉ

Les connexines sont indispensables à la glande mammaire, car elles permettent la communication intercellulaire entre les cellules dans les couches luminales et myoépithéliales de son épithélium. Il est bien documenté que l'expression des connexines est spécifique au stade de développement de la glande mammaire; cependant, leurs mécanismes de régulation, spécifiquement par les microARNs (miARNs), restent mal caractérisés.

L'hypothèse de ce projet était que l'expression des miARNs varient entre les stades de développement des glandes mammaires, et sont régulés par des hormones clés dirigeant sa morphogenèse. Il est également supposé que cette régulation est liée aux connexines, en particulier Cx43, et que les hormones et miARNs régulent fonctionnellement son ARNm. Basés sur ces hypothèses, nos objectifs étaient de mesurer l'expression des miARNs à différents stades de développement *in vivo*, d'identifier l'effet des hormones sur les miARNs et l'ARNm Cx43 *in vitro*, et de confirmer la suppression de l'ARNm de Cx43 par miARNs en modifiant leurs niveaux *in vitro*. Pour ce faire, nous avons extrait et séquencé les miARNs de a) glandes mammaires de souris à quatre étapes importantes de développement, et b) cellules cancéreuses luminales exposées à l'estradiol, la progestérone, la prolactine et l'ocytocine. Ces mêmes hormones ont également été utilisées lors d'expositions à court terme afin de déterminer leur effet sur l'ARNm de Cx43.

Il a été constaté que l'expression des différents miARNs varient considérablement tout au long du développement, mais aussi dans des groupes suivant des modèles d'expression similaires. Basé sur leur expression et sur leur régulation par les hormones, nous avons pu identifier des miARNs dont l'expression est vraisemblablement liée aux hormones circulantes de façon stade-dépendante. *In vitro*, l'estradiol et la progestérone ont augmenté l'ARNm de Cx43, et l'ocytocine a diminué l'ARNm de Cx43 en courtes périodes. De plus, la prolactine augmente l'expression de l'ARNm de Cx43 deux heures après l'exposition, mais finit par diminuer l'ARNm de Cx43 après huit heures. Ces mêmes hormones ont changé des groupes distincts de miARNs.

Il est intéressant de noter que les miARNs et Cx43 sont dérégulés pendant le cancer du sein d'une manière qui favorise le microenvironnement tumoral, favorisant la progression et les métastases. Le projet apportera ainsi des informations importantes sur le développement des glandes mammaires, mais servira également de prélude à d'autres études sur l'une des maladies les plus graves affectant les femmes dans le monde.

Mots clés: Connexine 43 (Cx43), microARN (miARN), hormone, junction lacunaire, communication intercellulaire, cancer

SYNOPSIS

Contexte du projet

La glande mammaire est essentielle car elle synthétise et secrète du lait, nourrissant la progéniture des mammifères femelles pour permettre leur croissance. Structurellement, la glande mature est composée d'un système de canaux épithéliaux ramifiés, entourés d'un stroma. Pendant la grossesse, l'épithélium prolifère et se différencie massivement, créant une glande fonctionnelle qui secrète du lait durant la lactation. Après la grossesse, il y a l'involution de la glande, période durant laquelle la glande mammaire régresse à une forme similaire à la pré-grossesse par apoptose et remodelage (Macias et al. 2012). La transition entre les différents stades de développement est dirigée principalement par les hormones estradiol (E_2), progestérone (P_4), prolactine (PRL) et ocytocine (OXT) (Macias et al. 2012; Lollivier et al. 2006). De plus, les cellules de la glande doivent communiquer pour assurer son développement normal. Les cellules dans les deux couches de l'épithélium, composées respectivement de cellules luminales et myoépithéliales, transfèrent des molécules directement entre elles via des jonctions lacunaires.

Les jonctions lacunaires sont constituées d'un assemblage en plaques de canaux bidirectionnels, chacun formé par l'assemblage de deux hemi-canaux, appelés connexons, provenant de cellules opposées. Individuellement, les connexons sont formés par l'hexamérisation des protéines transmembranaires appelées connexines. Les jonctions lacunaires permettent une forme de communication moléculaire requise pour le développement et la fonction de l'épithélium et de la glande mammaire, mais aussi pour la plupart des organes chez diverses espèces. L'expression des connexines est spécifique au stade de développement de la glande mammaire, liée aux hormones et régulée par divers mécanismes, notamment les facteurs de transcription, les modifications des histones, la méthylation de l'ADN, les variantes de traduction, les modifications post-traductionnelles et les interactions protéine-protéine (M. Oyamada et al. 2013; Aasen et al. 2019). Cependant, malgré leur rôle central dans le développement, la régulation des connexines par les microARNs (miARNs) est très peu étudiée. Les miARNs sont des ARNs courts et non codants qui régulent l'ARNm cible de manière post-transcriptionnelle, entraînant des changements de l'expression des gènes (Reichholf et al. 2019). Les objectifs de ce projet sont: 1) de caractériser les profils d'expression des miARNs à divers stades de développement de la glande mammaire chez la souris, 2) de déterminer l'impact des hormones sur a) le profil des

miARNs *in vitro* et b) l'expression de l'ARNm de Cx43, et enfin 3) de confirmer la régulation de Cx43 par certains miARNs en modifiant leurs niveaux *in vitro* (Figure 1.1).

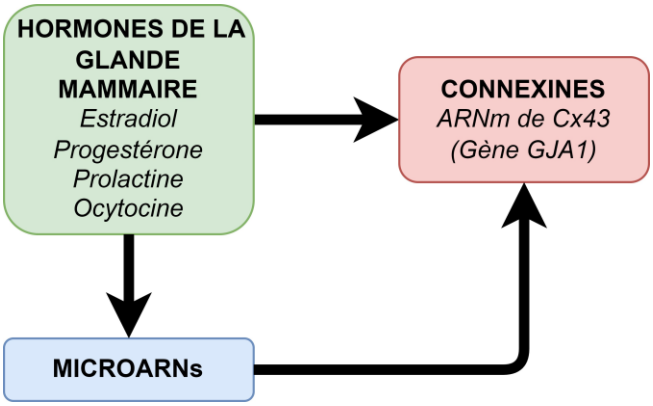

Figure 1.1: Résumé du projet. Ce projet vise à déterminer le rôle des microARNs dans le développement de la glande mammaire, leur induction ou inhibition par hormones de la glande mammaire, et leur régulation de l'expression de Cx43. Les miARNs sont des ARNs courts et non codants dont les effets sur l'homéostasie et la maladie sont largement inconnus.

Structure et développement de la glande mammaire

Comme d'autres organes, la fonction de la glande mammaire est lié a sa structure. Dans sa forme mature, la glande mammaire est constituée d'un épithélium sécrétoire entouré d'un stroma (Figure 1.2). L'épithélium est formé d'une couche basale extérieure composée principalement de cellules myoépithéliales et d'une couche interne de cellules luminales (Cyr et al. 2016), tandis que le stroma contient des cellules immunitaires, des fibroblastes et des adipocytes (Wiseman et al. 2002). L'épithélium forme un système de canaux galactophores qui terminent en lobes, qui contiennent de nombreux lobules (Figure 1.2) et d'acini qui produisent du lait (Macias et al. 2012). Les canaux galactophores s'écoulent au niveau du mamelon, qui permet la sortie du lait et l'alimentation de la progéniture des mammifères femelles.

La glande mammaire est unique, car son développement se produit principalement après la naissance dans un processus de plusieurs étapes (Macias et al. 2012). Au cours du développement, la glande mammaire passe d'un état inactif aux stades dynamiques de la grossesse, la lactation et l'involution. La grossesse est une phase de prolifération et de différenciation de l'épithélium mammaire qui conduit à la formation des alvéoles, qui sont les structures qui sécrètent le lait (Macias et al. 2012). Pendant la lactation, les alvéoles produisent une quantité importante de lait, ce qui augmente leur taille et augmente l'occupation de l'espace dans le stroma environnant (Figure 1.3) (Macias et al. 2012). Lors de l'allaitement de la

progéniture, la couche de cellules myoépithéliales entourant les alvéoles se contracte, provoquant la libération de lait des cellules luminales internes, qui traverse les canaux galactophores jusqu'au mamelon (Hannan et al. 2023). Enfin, lors de l'involution, la glande mammaire retourne à une forme similaire à la pré-grossesse par apoptose.

Hormones clés de la glande mammaire impliquées dans son développement

Le remodelage à grande échelle associé au développement de la glande mammaire nécessite de grandes modulations de l'expression des gènes. Les changements morphologiques des compartiments sont médiés en grande partie par l'estradiol (E_2), la progestérone (P_4), la prolactine (PRL) et l'ocytocine (OXT) (Macias et al. 2012; Lollivier et al. 2006). E_2 est une hormone synthétisée principalement dans les ovaires, et est la forme la plus impliquée dans la reproduction et le développement (Rusidzé et al. 2021) par rapport aux trois autres formes, l'estrone, l'estrol et l'estetrol (Cui et al. 2013; Gérard et al. 2015). Pendant la puberté, E_2 agit conjointement avec l'hormone de croissance et le facteur de croissance de type insuline 1 (IGF1) afin de stimuler une augmentation de la morphogenèse canalaire, augmentant le volume occupé par l'épithélium (Gallego et al. 2001; Ruan et al. 1999; Macias et al. 2012). P_4 est une hormone stéroïde paracrine dont la structure est semblable à celle de l'E_2 et qui est principalement produite dans les ovaires (Macias et al. 2012). Cependant, elle n'est pas nécessaire aux mêmes périodes que E_2, car l'inactivation génique de son récepteur (PR) ne change pas le phénotype mammaire pendant la puberté (Lydon et al. 1995). Par contre, la signalisation par PR est nécessaire pour la ramification tertiaire et l'alvéogenèse (Lydon et al. 1995; Brisken et al. 1998).

PRL est une hormone peptidique sécrétée par l'hypophyse antérieure et régulée par l'hypothalamus, mais aussi produite dans la glande mammaire (Macias et al. 2012; Freeman et al. 2000). Pendant la grossesse, la PRL contribue à la production d'alvéoles et pendant la lactation, elle permet de conserver la structure complexe de la glande mammaire (Macias et al. 2012). Finalement, OXT est une hormone peptidique la plus active à la lactation (Lollivier et al. 2006). Sa sécrétion est déclenchée par la succion de la progéniture, qui provoque la transmission d'impulsions nerveuses à l'hypothalamus, provoquant la sécrétion d'OXT de l'hypophyse postérieure et la liaison à son récepteur (OXTR) sur les cellules myoépithéliales, illicitant leur contraction (Gimpl et al. 2001; Lollivier et al. 2006).

Implication des jonctions lacunaires dans le développement de la glande mammaire

En plus des hormones, le développement de la glande mammaire est médié par les jonctions lacunaires (Stewart et al. 2015; El-Sabban et al. 2003). La communication intercellulaire par jonctions lacunaires est un processus critique, car elle permet une communication moléculaire directe entre les cellules, coordonnant entre autres, la croissance, la division et la différenciation (Goodenough et al. 2009; Shaw et al. 2007; Zefferino et al. 2019; Delmar et al. 2018). Les jonctions lacunaires sont formées de canaux bidirectionnels composés de protéines transmembranaires appelés connexines (Stewart et al. 2015). Les connexines sont transcrites à partir de divers gènes dont GJA1 pour la connexine 43 (Cx43) (Oyamada et al. 2013). Ces protéines s'organisent en hexamères, formant l'hemicanal, ou connexon (Laird 2006). Deux hémicanaux de membranes opposées s'assemblent pour former des canaux bi-directionels, qui se concentrent dans des plaques, formant les jonctions lacunaires matures (Laird 2006). Les jonctions lacunaires sont présentes dans presque tous les tissus, mais elles diffèrent selon le type de tissu, car les connexines exprimées et constituant les jonctions lacunaires sont différentes (Goodenough et al. 2009). Ces structures importantes permettent le transfert direct de molécules d'environ 1 kilodalton (kDa) entre des cellules adjacentes (Stewart et al. 2015). Le Tableau 1.1 présente des types de molécules et des exemples pour chaque type, qui sont perméables aux jonctions lacunaires ou aux hémicanaux.

Tout au long des stades de développement de la glande mammaire murine femelle, l'expression de Cx43 reste élevée, mais atteint un minimum bien défini dans les jours 7 à 14 de la lactation (Dianati et al. 2016). Les variations de l'expression des connexines (Cxs) à divers étapes de développement suggèrent qu'elles jouent des fonctions importantes spécifiques au stade de développement (Stewart et al. 2015). Leur expression est également corrélée avec l'expression des hormones de la glande mammaire; par exemple, la lactation est caractérisée par une élévation de la PRL et l'OXT et dimimuation de E_2 et P_4, tandis que Cx43 est exprimé au minimum (Dianati et al. 2016). Il est donc postulé que la régulation des Cxs peut être liée aux voies activées ou désactivées par diverses hormones tout au long du développement. Dans la glande mammaire, il a été observé que les souris exprimant une mutation de Cx43 présentent une diminution de communication intercellulaire par jonctions lacunaires (Plante et al. 2008). Les épithéliums des glandes mammaires dans cette étude étaient caractérisés par un retard de développement et une incapacité à sécréter du lait. Ainsi, le manque de Cx43 entraîne un retard de développement et une perte de fonction de la glande mammaire.

Régulation de l'expression de Cx43 : exemples

Il a été démontré que Cx43 a un court cycle de vie de quelques heures (Epifantseva et al. 2018). Les niveaux de Cx43 et la communication intercellulaire par jonctions lacunaires peuvent être rapidement augmentés ou diminués en fonction des besoins des tissus (Laird et al. 2006). Par exemple, une expression fortement accrue de Cx43 a été observée dans l'utérus de souris au début de la parturition, permettant probablement une contraction synchronisée des muscles de l'utérus pendant l'accouchement (Tong et al. 2009). Il a été démontré que Cx43 peut être régulé aux niveaux transcriptionnels et post-transcriptionnels (M. Oyamada et al. 2013). Par exemple, la phosphorylation est l'une des modifications post-traductionnelles les plus largement étudiées et impactantes de Cx43, régulant sa stabilité à la membrane plasmique, la formation d'hexamères, la dégradation, la perméabilité des canaux et les interactions protéine-protéine (Tableau 1.2, Figure 1.9) (Lampe et al. 2004; Solan et al. 2020). De plus, la phosphorylation par les kinases agissant sur Cx43 comme PKA, PKC et MAPK, entre autres (Lampe et al. 2004), peut se produire à de nombreux sites différents. D'autres facteurs influançant l'expression de Cx43 incluent les modifications d'histones, la méthylation de l'ADN, les facteurs de transcription, l'épissage alternatif, la SUMOylation, l'ubiquitination, les interactions protéine-protéine, et bien entendu, les hormones (Figure 1.9) (M. Oyamada et al. 2013; Aasen et al. 2019).

Régulation de Cx43 par miARNs

Au cours des dernières années, un nombre croissant d'études a démontré que l'expression des Cxs peut être régulée par des miARNs (Figure 1.10) dans divers tissus, mais il existe peu d'information sur le rôle des miARNs dans la régulation de Cx43 pendant le développement de la glande mammaire, et comment les hormones contribuent à cette régulation. Des exemples de régulation de Cx43 par miARNs dans divers tissus et organes sont présentés dans le Tableau 1.3.

Dans les cellules cancéreuses du sein, miR-1, miR-206, miR-200a, miR-381, miR-23a, miR-23b et miR-186 ont été identifiés comme inhibiteurs fonctionnels de l'expression de Cx43 (Ming et al. 2015a). Des essais de luciférase ont montré que miR-200a-3p est capable de cibler Cx43 directement à la région non-traduite 3' de son ARNm, et qu'il était également impliqué dans la migration des cellules MCF7 (Ming et al. 2015a). Dans une autre étude, l'augmentation de miR-206 a été associée à une diminution de la migration et de l'invasion des cellules MDA-MB-231 et

MDA-MB-436 en ciblant directement l'ARNm de Cx43 à la région non-traduite 3' (Fu et al. 2015). D'autres exemples seront démontrés plus bas.

Régulation des miARNs par les hormones de la glande mammaire

Les hormones sont des régulateurs importants du développement et de la fonction des tissus. Normalement, les hormones se liant à leur récepteur induiront des voies de signalisation, ayant comme résultat l'expression de nombreux gènes. Étant donné le rôle des miARNs dans la régulation des gènes, on peut supposer que les hormones réguleront également l'expression des miARNs, contribuant ainsi à la réponse cellulaire et tissulaire. Quelques études ont analysé cette relation, y compris dans la glande mammaire et les cellules cancéreuses du sein. Par exemple, l'effet d'un traitement des cellules cancéreuses du sein MCF7 et ZR-75.1 avec 10 nM E_2 a été évalué à l'aide de microréseaux (Ferraro et al. 2012). L'expression de 52 miARNs communs ont été modulés entre les deux lignées cellulaires, et des dizaines d'autres ont été modifiées dans une des deux lignées cellulaires. D'autre part, il a été révélé de façon surprenante que 10 nM E_2 n'affectait pas l'expression des miARNs dans les cellules T-47D après 24 heures (Katchy et al. 2012).

Liens entre Cx43, les miARNs et le cancer du sein

Le développement normal de la glande mammaire dépend de la communication intercellulaire par jonctions lacunaires; pour cette raison on croyait originalement que les connexines étaient des suppresseurs de tumeurs. Cependant, les recherches ultérieures indiquent que leur rôle est beaucoup plus complexe, dépendant du sous-type et du stade du cancer du sein. Ce projet se concentre sur Cx43 parce que son rôle dans la biologie de la glande mammaire est crucial et il peut aussi contribuer au développement du cancer du sein (Chasampalioti et al. 2018; Busby et al. 2018). Parmi les quatre types intrinsèques de cancer du sein, souvent les types luminal A, luminal B, basal et le Her2e, il a été démontré que Cx43 est sur-exprimé dans les tumeurs non-ratifiées et luminales, mais sous-exprimé dans le sous-type Her2e (Busby et al., 2018). Cx43 peut agir comme suppresseur de tumeur, car la diminution de son expression peut entraîner une communication intercellulaire aberrante et une progression accrue du cancer (Fentiman et al. 1977; Nicolas et al. 1978), mais aussi comme oncogène dont la surexpression est associée à la formation de métastases (el-Sabban et al. 1994; Elzarrad et al. 2008). En général, le rôle des miARNs dans la progression du cancer du sein est similaire à celle de Cx43. Comme les miARNs régulent des processus tels que la prolifération, la différenciation, l'apoptose et la migration, il

n’est pas surprenant que des dizaines de miARNs ont été découverts comme associés au cancer du sein (Loh et al., 2019; Davey et al., 2021). Les miARNs sont eux-mêmes transcrits à partir de gènes, donc la sur-activation ou l’inhibition des mécanismes de régulation des gènes peuvent entraîner une augmentation ou une suppression de l’expression de leurs protéines cibles, provoquant la croissance tumorale. La plupart des rôles mécanistiques des miARNs dans le cancer du sein sont inconnus, mais comme Cx43, ils peuvent jouer le rôle de suppresseurs de tumeur ou d’oncogènes.

Article #1

En raison de leur association avec le cancer du sein, il est particulièrement important de comprendre le rôle des miARNs dans le développement de la glande mammaire et dans la régulation de Cx43 afin de déterminer comment ils peuvent contribuer à sa pathogenèse. Les objectifs de l’article #1 sont les suivants : 1) Analyser les profils d’expression des miARNs à différents stades du développement des glandes mammaires *in vivo*; 2) Déterminer l’impact des hormones sur le profil des miARNs *in vitro* ; 3) Évaluer si la variation des hormones entre les stades de développement pourrait être corrélée avec des groupes spécifiques de miARNs; et 4) Prédire les cibles génétiques potentielles des miARNs exprimé et régulés par les voies hormonales *in silico*.

L’hypothèse de cet article est la suivante: les microARNs sont exprimés de manière différentielle en fonction des stades de développement de la glande mammaire chez la souris, et sont étroitement liés à l’expression des hormones associées aux stades de développement de la glande. Nous prévoyons également que les cibles des miARNs différentiellement exprimés et régulés par les hormones sont associées à la transition entre les stades et les voies hormonales, respectivement.

Pour atteindre les objectifs de cet article, des glandes mammaires ont été récoltées de six souris aux stades de développement suivants : semaine 10 (S10), jour huit de la grossesse (G18), jour sept de la lactation (Lac7) et jour trois de l’involution (Inv3) (N = 6), et converties en poudre dans des conditions cryogéniques. Ensuite, l’extraction et le séquençage des miARNs ont été effectués sur les échantillons. De plus, des cellules T-47D, une lignée de cellules cancéreuses du sein luminales provenant d’une patiente de 54 ans, ont été cultivées et traitées avec E_2, P_4, PRL et OXT. Ces cellules sont moins aggressives que les cellules triple négatives du cancer du sein. Elles sont aussi un bon modèle de la communication intercellulaire par jonctions lacunaires dans

la glande mammaire, car elles contiennent les récepteurs des hormones (E_2, P_4, PRL et OXT) impliqués dans le développement de la glande mammaire, et l'expression de Cx43 (Fay et al. 1999; Yu et al. 2017; Leehy et al. 2018; Wu et al. 2021). Lors de la première série d'expositions, des protéines ont été extraites et des marqueurs ont été quantifiés par analyse Western, afin de valider l'effet des hormones sur les cellules. Au cours de la deuxième série d'expositions, des miARNs ont été extraits et séquencés (N = 3), afin d'évaluer l'effet des hormones sur le profil des miARNs dans les cellules cancéreuses du sein. Pour les expériences de séquençage *in vivo* et *in vitro*, la qualité de tous les extraits a été validée à l'aide d'un spectrophotomètre Nanodrop et d'un bioanalyseur 2100. Le Nanodrop mesure la concentration des acides nucléiques en fonction de l'absorbance de la solution. De plus, des anormalités d'absorbance de la solution pointent vers divers contaminants, ce qui donne des informations sur la pureté de la solution. Le bioanalyseur mesure les émissions de fluorescence des solutions d'acides nucléiques sur une puce contenant les parties d'un gel d'acides nucléiques régulier, mais sous forme miniaturisée. La taille de divers acides nucléiques est visible, ce qui donne de l'information sur l'intégrité des acides nucléiques dans la solution.

Article #2

L'article #1 a démontré que l'expression des miARNs varie selon les différents stades de développement de la glande mammaire (Article #1, Figure 1, page 39), collectivement sous forme de groupes, et aussi individuellement. De plus, les miARNs régulés lors de l'exposition à E_2, P_4, PRL et OXT ont été identifiés dans les cellules T-47D. Il a également été trouvé que leur expression est liée à de nombreuses voies régulées par les hormones ou associées à la transition entre les stades. Cet article nous a permis d'identifier des miARNs associés aux hormones qui régulent potentiellement le développement de la glande mammaire. Cependant, la régulation de l'expression de Cx43 par les miARNs, et la façon dont ils peuvent contribuer à la communication intercellulaire par jonctions lacunaires, n'étaient pas encore déterminées. Des analyses supplémentaires étaient donc nécessaires a) pour prédire quels miARNs pourraient réguler Cx43 *in silico*; b) pour lier l'expression des miARNs avec l'expression de l'ARNm de Cx43; c) pour analyser comment les hormones affectent l'ARNm de Cx43; et d) pour confirmer si les niveaux d'ARNm de Cx43 sont modulés par les miARNs.

Les objectifs de l'article #2 sont les suivants: 1) Prédire quels miARNs pourraient réguler Cx43 en utilisant des bases de données en ligne; 2) Identifier les candidats qui sont inversement corrélés à l'expression de l'ARNm de Cx43 et influencés par la présence des hormones; 3) Tester

la régulation de l'ARNm Cx43 par ces mêmes hormones; et 4) Confirmer l'effet de certains candidats sur les niveaux de l'ARNm de Cx43, en fonction des résultats des objectifs précédents.

L'hypothèse de cet article est la suivante: certains candidats *in silico* sont inversement corrélés à Cx43 et liés à des hormones, suggérant une régulation des miARNs de ses niveaux d'ARNm, d'une manière hormono-dépendante. Nous prévoyons également qu'un ou plusieurs de ces candidats suppriment fonctionnellement l'ARNm de Cx43.

Pour atteindre les objectifs de cet article, des bases de données en ligne ont été utilisées pour rechercher des miARNs potentiellement régulant l'expression de Cx43. L'expression de chaque miARN *in vivo* et *in vitro* lors de l'exposition aux hormones a été analysée pour identifier des candidats qui sont inversement corrélés avec ses niveaux d'ARNm et associés aux hormones. Afin d'évaluer l'effet des hormones sur l'ARNm de Cx43, les cellules T-47D ont été exposées à E_2, P_4, PRL et OXT pendant 0 à 8 heures. Ensuite, l'extraction de l'ARN total, la transcription inverse et des expériences qPCR ont été effectuées afin de mesurer l'ARNm de Cx43. Pour confirmer l'effet des miARNs sur l'expression de Cx43, des mimétiques et inhibiteurs des miARNs, qui augmentent et diminuent des miARNs spécifiques respectivement, ont été transfectés dans les cellules T-47D. Ensuite, le changement de l'ARNm de Cx43 à été évalué comme ci-dessus.

Résultats principaux

Dans notre premier article, nous avons montré que 490, 473, 419 et 460 miARNs sont détectés dans la glande mammaire chez les souris adultes, gestantes, allaitantes et subissant une involution, respectivement (Article 1, Figure 2). La plupart d'entre eux étaient communs aux quatre groupes, mais 58 étaient uniques à un stade. La plupart des gènes pourraient être divisés en six groupes d'expression, dont deux contenaient le plus grand nombre de miARNs (groupes 1 et 3) et présentant des profils d'expression opposés, atteignant un maximum à l'âge adulte et un minimum à la lactation, ou minimum à l'âge adulte et maximum à la lactation (Article 1, Figure 3). Des analyses GO et KEGG suggèrent que les miARNs différemment exprimés entre les stades influencent l'expression des cibles associées à l'homéostasie et la fonction des glandes mammaires, et à la régulation hormonale (Article 1, Figures 4 à 6). Pour mieux comprendre les liens entre l'expression des miARNs et les hormones impliquées dans le développement des glandes mammaires, les miARNs ont ensuite été séquencés dans des cellules du sein exposées à E_2, P_4, PRL et OXT. 38, 4, 24 et 66 miARNs étaient associés à une exposition à E_2, P_4, PRL et

OXT, respectivement (Article 1, Figure 7). Enfin, lors de l'analyse des miARNs modulés par les hormones, différemment exprimés au cours du développement et ayant un modèle d'expression potentiellement corrélé avec les niveaux relatifs d'hormones *in vivo*, 16 miARNs ont été identifiés comme étant probablement régulés par les hormones circulantes (Article 1, Figure 1; 8 à 15).

Dans la deuxième étude, nous avons cherché à élucider les liens entre les miARNs, les hormones et la régulation de Cx43. Les miARNs liés aux hormones potentiellement régulant l'expression de l'ARNm de Cx43 ont été identifiés en considérant a) la prédiction des interactions ARNm-miARN à l'aide de bases de données en ligne, b) la corrélation inverse entre les miARNs et l'ARNm de Cx43 *in vivo*, c) la modification des profils des miARNs lors de l'exposition aux hormones *in vitro*, et d) les effets de ces mêmes hormones sur l'expression *in vitro* de l'ARNm de Cx43. Cela a permis d'identifier neuf miARNs inversement corrélés et sept positivement corrélés avec l'expression de l'ARNm de Cx43 (Article 2, Figures 1 et 2) (Dianati et al. 2016). De plus, trois, zero, quatre et cinq de ces miARNs étaient liés à l'exposition à l'estradiol, la progestérone, la prolactine et l'ocytocine, respectivement (Article 2, Figure 3). L'expression de l'ARNm de Cx43 a augmenté en réponse à une exposition à E_2, P_4 et PRL pendant 2 h, et a diminué suite à l'exposition à l'OXT et PRL pendant 8 h (Article 2, Figure 4). Comme l'expression des miARNs est généralement associée à une diminution de l'expression des cibles d'ARNm, ces résultats suggèrent que la régulation négative de miR-125b-5p et miR-30c-5p par E_2 peut être associée à une expression accrue de Cx43. De même, ces deux miARNs pourraient potentiellement avoir un effet à court terme sur l'ARNm Cx43 lors d'une exposition à PRL, car son expression est diminuée après 2 h d'exposition. De même, l'OXT peut réguler l'expression de quatre miARNs, entraînant une diminution globale de l'expression du Cx43. Bien que ces miARNs semblent être importants pour la régulation de Cx43, plus d'études sont nécessaires pour comprendre l'interaction complexe reliant les hormones et les miARNs à Cx43 pendant le développement de la glande mammaire.

Discussion

Les données sur l'expression différentielle de miARNs *in vivo* suggèrent que, comme les protéines, les miARNs sont omniprésents dans la glande mammaire. Des groupes de miARNs ont été identifiés comme possédant des motifs d'expression similaires au cours du développement (par exemple, des groupes dont les miARNs atteint un maximum à des points de temps similaires). Cependant, des miARNs pourraient être exprimés à des magnitudes différentes ou diverger des groupes identifiés, démontrant des modèles d'expression individuels distincts. Il

est donc suggéré que les miARNs jouent des fonctions importantes et étendues. En examinant les profils d'expression des miARNs individuels *in vivo*, certains miARNs sont probablement indispensables en tant qu'individus ou familles au développement et fonction des glandes mammaires. Par exemple, dans notre étude, les cinq membres de la famille miR-200 (Humphries et al. 2015) ont affiché une expression maximale à la lactation. De G18 à Lac7, mmu-miR-200a-3p, 200b-3p, 141-3p et 429-3p ont augmenté par des facteurs de 2, 2, 4 et 3, respectivement. Ils faisaient aussi partie des 30 gènes les plus différentiellement exprimés de G18 à Lac7 et de Lac7 à Inv3. Dans l'étude de Xuan et al. 2020, l'expression différentielle de chi-miR-200b-3p et de chi-miR-200c-3p en fin de lactation par rapport aux deux autres stades a été identifiée, tandis que Li et al. 2012b ont identifié l'expression de miR-200a-3p comme maximale à la lactation par rapport aux autres stades. Notre étude soutient la forte expression de la famille miR-200 à la lactation démontrée dans la littérature et suggère un rôle important de ce miARN dans le fonctionnement de la glande mammaire.

Des miARNs qui ne jouent que des fonctions mineures peuvent ne pas être nécessaires au développement des glandes mammaires, mais la perte collective de ces miARNs pourrait encore entraîner un développement délétère. La régulation de Cx43 par les miARNs est affectée par une mosaïque de facteurs régulateurs. À tout moment, un ou plusieurs de ces facteurs peuvent être eux-mêmes régulés différemment, globalement par les hormones, ou individuellement aux niveaux transcriptionnel, post-transcriptionnel, traductionnel ou post-traductionnel. Ainsi, la régulation de Cx43 par les miARNs n'est pas un processus statique, mais est étroitement et dynamiquement liée à d'autres voies cellulaires.

Ce projet s'est concentré sur le rôle des miARNs dans le développement normal des glandes mammaires; cependant, une partie utilise des cellules T-47D, une lignée cellulaire cancéreuse luminale, ce qui met en lumière l'expression des miARNs dans l'état du cancer du sein. Si les miARNs qui sont normalement des suppresseurs de tumeurs sont sous-régulés dans les cellules cancéreuses du sein, ces miARNs pourraient contribuer aux propriétés de la maladie. D'autre part, si les miARNs connus comme oncogènes sont inhibés dans les cellules cancéreuses du sein, ces miARNs pourraient également avoir un effet sur les propriétés de la maladie. Les miARNs exprimés de façon aberrante et associés à Cx43 pourraient contribuer à la communication intercellulaire par jonctions lacunaires anormale observée dans de nombreux cancers (Holder et al. 1993; Cronier et al. 2009; Aasen et al. 2017; Busby et al. 2018). Le Tableau 1.4 présente des exemples de miARNs qui ont montré une expression différentielle dans les glandes mammaires de souris par rapport aux cellules cancéreuses du sein.

Perspectives et conclusions

Cette étude ajoute des connaissances sur l'importance des miARNs, qui régulent environ un tiers du protéome (Macfarlane et al. 2010), dans le développement de la glande mammaire. Contrairement à d'autres articles, ce projet a analysé le profil des miARNs à de nombreux stades de développement de la souris. Ceci a mené à l'identification de groupes distincts de miARNs avec grandes fluctuations au cours du développement, plutôt qu'un profil à une étape unique. Les données sont disponibles aux chercheurs(euses) étudiant non seulement la communication intercellulaire par jonctions lacunaires, mais d'autres processus cellulaires, car la méthode de miRNA-seq identifie l'expression globale des miARNs. La confirmation de l'expression des miARNs au niveau individuel est particulièrement importante parce qu'il existe peu d'études sur le séquençage des miARNs dans la glande mammaire. Le projet apporte aussi de l'information sur l'effet de E_2, P_4, PRL et OXT sur le profil des miARNs dans les cellules cancéreuses du sein. Contrairement aux recherches axées sur une hormone seulement, cette étude a combiné des expériences *in vivo* avec des expériences *in vitro* sur les hormones, mettant en lumière leurs effets sur le profil des miARNs. Différentes conditions ont été sélectionnées afin de tenir compte des différentes concentrations physiologiques d'hormones et permettre une comparaison avec la littérature limitée.

Les études de suivi potentielles pourraient viser d'abord à confirmer si les altérations de l'ARNm de Cx43 par les miARNs entraînent des effets sur les niveaux protéiques de Cx43. Ceci est pertinent, car les changements d'expression de l'ARNm ne se traduisent pas toujours par changements aux niveaux protéiques, et ce sera finalement la protéine Cx43 qui participera à la communication intercellulaire par jonctions lacunaires. Ceci serait accompli en effectuant des transfections d'ARNs mimiques et inhibitrices des miARNs dans les cellules T-47D et en testant l'expression de Cx43 par analyse Western. La liaison directe des miARNs à la 3' UTR de l'ARNm pourrait être effectuée à l'aide d'un test de luciférase afin d'obtenir des preuves plus directes de la régulation de l'ARNm de Cx43 par les miARNs. D'autres miARNs dans notre liste de candidats peuvent être transfectés et testés pour des liens avec l'estradiol, la progestérone, la prolactine et l'ocytocine. Tels liens peuvent être testés avec d'autres connexines, pour élargir les connaissances sur l'effet des miARNs sur la communication intercellulaire par jonctions lacunaires. Enfin, en raison du fait que les jonctions lacunaires sont liées à d'autres protéines jonctionnelles, il serait intéressant de tester des liens entre les miARNs et d'autres protéines jonctionnelles.

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LIST OF ABBREVIATIONS

ADAM17 = Metalloproteinase domain containing protein 17

AGO = Argonaute

AMPK = AMP-activated protein kinase

AP-1 = Activator protein 1

AREG = Amphiregulin

ATP = Adenosine diphosphate

ATP = Adenosine monophosphate

ATP = Adenosine triphosphate

BMEC = Bovine mammary epithelial cell

C/EBPα = CCAAT/enhancer-binding protein α

cAMP = Cyclic AMP

circRNA = Circular RNA

Cx43 = Connexin 43

Cxs = Connexins

DE = Differentially expressed

DNMT3a = DNA (cytosine-5)-methyltransferase 3A

E/P/c = E_2, P_4 and cAMP

E_2 = Estradiol

EB1 = End-binding 1

EGFR = Epidermal growth factor receptor

ER = Estrogen receptor

ERα = Estrogen receptor alpha

ERβ = Estrogen receptor beta

FBS = Fetal bovine serum

FEN1 = Flap structure-specific endonuclease 1

FGF = Fibroblast growth factor

GJ = Gap junction

GJA1 = Gap junction alpha 1

GJIC = Gap junction intercellular communication

GO = Gene ontology

HRPT1 = Hypoxanthine-guanine phosphoribosyltransferase 1

Inv3 = Involution day 3

IP_3 = Inositol triphosphate

kDa = Kilodalton

KEGG = Kyoto Encylopedia of Genes and Genomes

Lac7 = Lactation day 7

MAPK = Mitogen-activated protein kinase

miR = miR strand

miR* = miR star strand

miRNA = MicroRNA

mRNA = Messenger RNA

OXT = Oxytocin

OXTR = Oxytocin receptor

P18 = Pregnancy day 18

P_4 = Progesterone

PAR-1 = Protease-activated receptor 1

PCNA = Proliferating cell nuclear antigen

PKA = Protein kinase A

PKC = Protein kinase C

PR = Progesterone receptor

PRA = Progesterone receptor A

PRB = Progesterone receptor B

Pre-miRNA = Precursor miRNA

Pri-miRNA = Primary miRNA

PRL = Prolactin

PRLR = Prolactin receptor

RANK = Receptor activator of NFKB1

RANKL = Receptor activator of NFKB1 ligand

RISC = RNA-induced silencing complex

RPL13A = Ribosomal protein L13a

RPM = Reads per million

RT-qPCR = Quantitative reverse transcription polymerase chain reaction

siRNA = Small interfering RNA

SIRPA = Signal regulatory protein alpha

SOCS1 = Suppressor of cytokine signaling 1

Sp-1 = Specificity protein 1

Sp-3 = Specificity protein 3

STAT5 = Signal transducer and activator of transcription 5

SUMO = Small ubiquitin-related modifier

TEB = Terminal end bud

UTR = Untranslated region

UXT = Ubiquitously Expressed Prefoldin Like Chaperone

W10 = Week 10 of age

WAP = Whey acidic protein

Wnt = Wingless/Integrated

Wnt4 = Wingless/Integrated 4

ZEB1 = Zinc finger E-box-binding homeobox 1

ZEB2 = Zinc finger E-box-binding homeobox 2

CHAPTER 1 : GENERAL INTRODUCTION

The mammary gland is essential because its synthesis and secretion of milk nourishes the offspring of female mammals, enabling their growth. Structurally, the mature gland consists of a system of branched, epithelial ducts surrounded by a supportive stoma. During pregnancy, the epithelium proliferates and differentiates massively, resulting in a functional milk-secreting gland present at lactation. This is followed by involution, whereby the mammary gland regresses to its pre-pregnancy form through apoptosis (Macias et al. 2012). Transition between various stages of development is orchestrated in large majority by the hormones estradiol (E_2), progesterone (P_4), prolactin (PRL) and oxytocin (OXT) (Macias et al. 2012; Lollivier et al. 2006). In addition, cells within the gland must communicate to ensure its proper development. Among others, cells within the two layers of the epithelium, comprised of luminal and myoepithelial cells respectively, transfer molecules directly between each other via gap junctions.

Gap junctions consist of an assembly in plaques of bidirectional channels, each formed by docking of two connexon hemichannels from opposing cells. Individually, connexons are formed by hexamerization of connexin transmembrane proteins. The molecular communication enabled by gap junctions is required not only for the mammary gland, its epithelium and their function, but also most organs in various species. Connexins' expression in the mammary gland is stage-specific, hormone-linked, and regulated by various mechanisms including transcription factors, histone modifications, DNA methylation, translation variants, post-translational modifications and protein-protein interactions (M. Oyamada et al. 2013; Aasen et al. 2019). However, despite their central role in development, the regulation of connexins by recently discovered microRNA (miRNA) is minimally researched. miRNA are short, non-coding RNA that post-transcriptionally downregulate target mRNA, resulting in changes in gene expression (Reichholf et al. 2019). The goals of this project are to 1) characterize miRNA expression profiles at various stages of mouse mammary gland development *in vivo*, 2) determine the impact of hormones on a) the miRNA profile and b) Cxs' mRNA expression, and lastly, 3) confirm the regulation of Cxs by select miRNA by altering their levels *in vitro* (Figure 1.1).

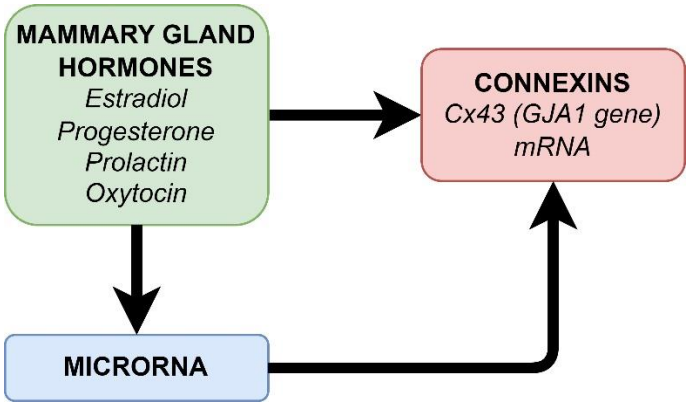

Figure 1.1: Project Introductory Figure. This project aims to determine the role of microRNA in mammary gland development, whether or not they are induced or inhibited by major hormones of the mammary gland, and their regulation of Cx43 expression. miRNA are short, non-coding RNA whose effects on homeostasis and disease are largely unknown.

1.1 Mammary gland structure, development and function

1.1.1 Mammary gland structure

Like other organs, the function of the mammary gland is dependent on its structure. However, this gland is unique as its development occurs mostly post-natally in a multistage process (Macias et al. 2012). In its mature form, the mammary gland consists of a secretory epithelium surrounded by a supportive stroma (Figure 1.2). The epithelium is formed by an outside basal layer mostly consisting of myoepithelial cells, and an inner layer of luminal cells (Cyr et al. 2016), whereas the stroma contains immune cells, fibroblasts and adipocytes (Wiseman et al. 2002). The epithelium forms a system of branching ducts ending in lobes, which contain many lobules (Figure 1.2) with numerous milk-producing acini (Macias et al. 2012). Ducts drain at the nipple, which serves as an outlet of milk that nourishes the offspring of female mammals during lactation. In humans, a single pair of mammary glands arise in the thoracic region (Khan et al. 2022), whereas in mice, five pairs of mammary glands exist, located in cervical, thoracic (two), abdominal and inguinal regions (Honvo-Houéto et al. 2015).

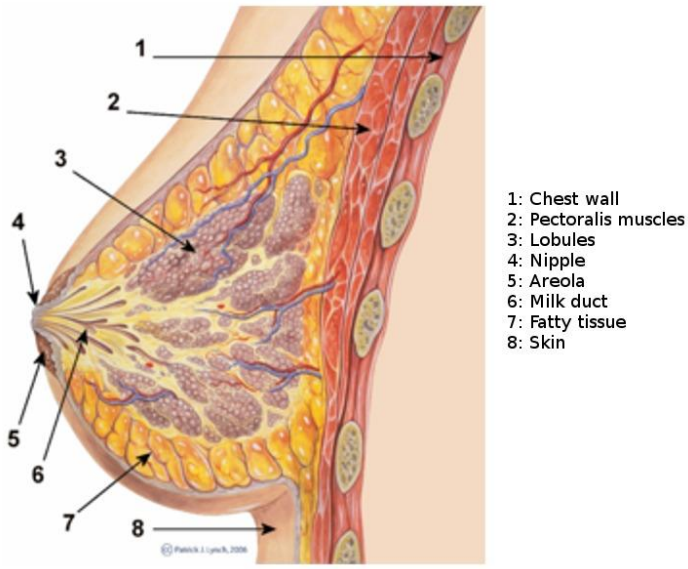

Figure 1.2: Breast structure. Mammary gland development occurs largely post-natally, resulting in a mature gland consisting of branching epithelial ducts surrounded by a supportive stroma. Diagram from Khan et al. 2022.

1.1.2 Embryonic and pre-pubertal development

In the developing embryo, the ectoderm proceeds to form the mammary epithelium, whereas the stroma originates from the mesoderm (Macias et al. 2012). Embryonic mouse mammary gland development has been reviewed by McNally et al. 2017. Briefly, development of placodes occurs at day 10 and 11 (mouse) along two mammary lines that run in the inguinal to cervical direction, which resolve into mammary buds that grow until day 15. Placodes are characterized as concentrated ectodermal areas of columnar cells. After a brief pause of development, buds elongate in days 15.5 and 16.5 via cellular proliferation, giving rise to a cord that proliferates into the fat pad precursor. The cord forms a lumen that terminates in the nipple, which will form by epidermal invagination. This rudimentary structure is visible as small glands on day 18.5 (Figure 1.3). The pre-pubertal gland consists of an elementary system of ducts minimally covering the stroma, in which fibroblasts and connective tissue can be found, and is largely quiescent in both mice and humans, growing at a similar pace to other body structures until puberty (McNally et al. 2017). In males, expression of the androgen receptor and sensitivity to testosterone (day 13) result in mesenchymal regression, leading to degradation of the epithelium in days 13.5 to 15.5.

1.1.3 Puberty

Beginning at around five weeks in mice and nine to twelve years in humans (McNally et al. 2017), puberty in the mammary gland consists largely of primary glandular branching morphogenesis, which results in rapid expansion of the initial mammary epithelium as well as a more complex ductal network (Figure 1.3) (Howlin et al. 2006). Cap cells and inner luminal cells of terminal end buds proliferating at the ends of ducts cause their expansion into the surrounding stroma (Macias et al. 2012). Primary ducts elongate and undergo bifurcation under the influence of stromal regulators, which act on terminal end buds, leading to additional primary ducts (Macias et al. 2012). The primary ducts further branch in the process of secondary branching. This complexified system continues growth up to about week eight when the edges of the stroma are reached (McNally et al. 2017). The terminal end buds then shrink and become mitotically inactive, with myoepithelial cells differentiating from cap cells (Macias et al. 2012). Tertiary branch development begins in response to cyclical ovarian stimulation, but will only be completed upon reaching pregnancy (Macias et al. 2012).

1.1.4 Pregnancy

Development of the epithelium as well as cellular proliferation and differentiation are dependent on receptor interactions with the extracellular matrix, a dynamic, protein-rich component of the stroma that links the two layers (Kass et al. 2007). Extracellular matrix remodeling is especially active in pregnancy, allowing major tissue remodeling, namely epithelial branch proliferation and development of alveolar buds (Macias et al. 2012). At the peak of this stage, myoepithelial cells surround luminal cells prominently, but partial contact of luminal cells with the extracellular is maintained, as necessary for alveolar differentiation (Fata et al. 2004). Alveolar buds differentiate from the tips of tertiary branches, forming distinct alveoli and eventually lobules (Macias et al. 2012). By day 18 of pregnancy, milk production is initiated by alveoli which become highly concentrated in the fat pad (Figure 31.) (McNally et al. 2017). Human mammary gland development during pregnancy is similar to mice, except lobules do not form during pregnancy, but are already present prior to this stage (McNally et al. 2017). The mammary gland is only considered fully differentiated in humans as the first pregnancy concludes (McNally et al. 2017).

1.1.5 Lactation

During lactation, substantial milk is produced by alveoli, resulting in their growth in size and further occupancy of space in the surrounding stroma (Figure 1.3) (Macias et al. 2012). Upon offspring suckling, the layer of myoepithelial cells surrounding alveoli contract, causing release of milk from inner luminal cells, which travels through ducts to the nipple and nourishes the offspring (Hannan et al. 2023). The process of milk release via suckling increases the production of more milk (Macias et al. 2012). Stromal fat metabolism catabolizes fats from adipocytes to support milk production, resulting in alveoli that are fully expanded into the stroma, a structural complexity which remains until weaning (McNally et al. 2017; Hovey et al. 2010).

1.1.6 Involution

Diminishing suckling of offspring results in an end to milk secretion and the beginning of regressive remodeling (Macias et al. 2012). The first part of involution is reversible (within two days in mice), as lactation can be re-initiated by resuming suckling (Li et al. 1997). If not re-initiated, programmed cell death results in decreased volume of the epithelium, whereby condensation of alveoli, re-expansion of stromal cells and immune cell influx occur (McNally et al. 2017). Also, extracellular matrix proteins begin degrading, which impacts involution advancement (McNally et al. 2017). Upon involution completion, the mammary gland resembles a pre-pregnancy level of complexity, with slightly more alveoli visible (Figure 3) (McNally et al. 2017). Future pregnancies re-initiate processes seen in the first, resulting in a re-expanded system of alveoli. With age, epithelial duct and alveolar complexity and function are reduced naturally, a process termed age-related involution (Macias et al. 2012).

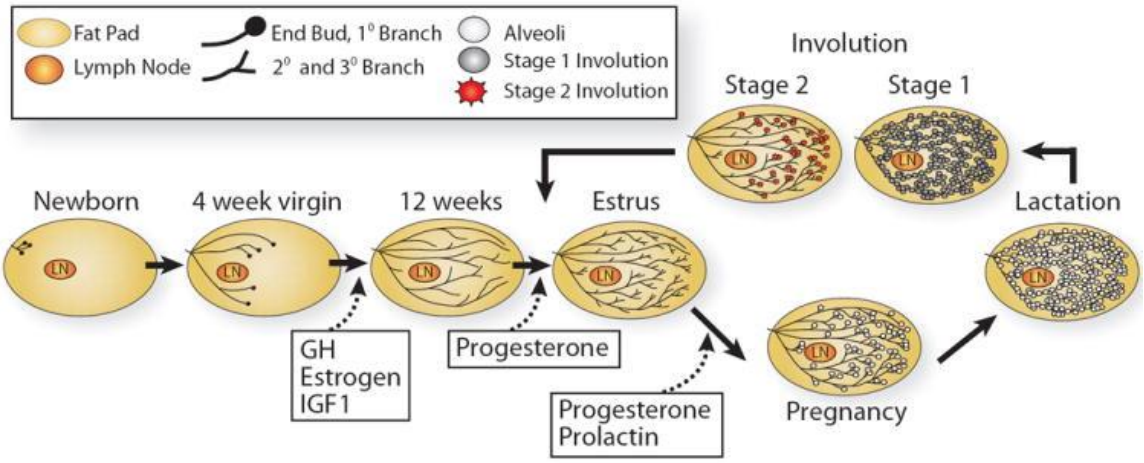

Figure 1.3: Stages of Mammary Gland Development in the Mouse. Mammary gland development occurs largely post-natally. Pregnancy is characterized by massive proliferation and differentiation of epithelium, leading to the formation of alveoli, the structures that secrete milk during lactation. This is followed by involution, whereby the mammary gland returns to its pre-pregnancy form via apoptosis. Figure from Macias et al. 2012.

1.2 Regulation of mammary gland development by hormones

Large-scale remodeling associated with each stage of development requires widespread transformation of gene expression. Morphology changes of various mammary gland compartments are mediated largely by hormones, more specifically estradiol, progesterone, prolactin and oxytocin.

1.2.1 Estradiol

E_2 is a membrane-soluble hormone synthesized mostly in the ovaries, and is the most potent form of estrogen involved in reproduction and mammary gland development (Rusidzé et al. 2021) compared with the other three forms, estrone, estrol and estetrol (Cui et al. 2013, Gérard et al. 2015). Its two receptors, estrogen receptor aplha (ERα) and estrogen receptor beta (ERβ), are responsive to E_2, though ERα is thought to be most involved in mammary development compared with ERβ, as knockout of this isoform results in impaired ductal development (Mallepell et al. 2006, Antal et al. 2008). During puberty, E_2 acts in conjunction with growth hormone and insulin-like growth factor 1 (IGF1) to stimulate a surge in ductal morphogenesis, increasing the volume of stroma occupied by the epithelium (Gallego et al. 2001, Ruan et al. 1999, Macias et al. 2012). Activated ERα induces amphiregulin (AREG) transcription, the membrane form of which is

cleaved by metalloproteinase domain containing protein 17 (ADAM17) of luminal cells, resulting in epidermal growth factor receptor (EGFR) activation in stromal cells, inducing fibroblast growth factor (FGF) release (Figure 1.4) (Rusidzé et al. 2021; Sternlicht et al. 2005). FGF then drives the proliferation of mammary epithelial cells, resulting in ductal elongation (Lu et al. 2008). E_2 also participates in alveogenesis during pregnancy, though mostly indirectly through induction of P_4 and PRL receptor (PR and PRLR) expression (Rusidzé et al. 2021).

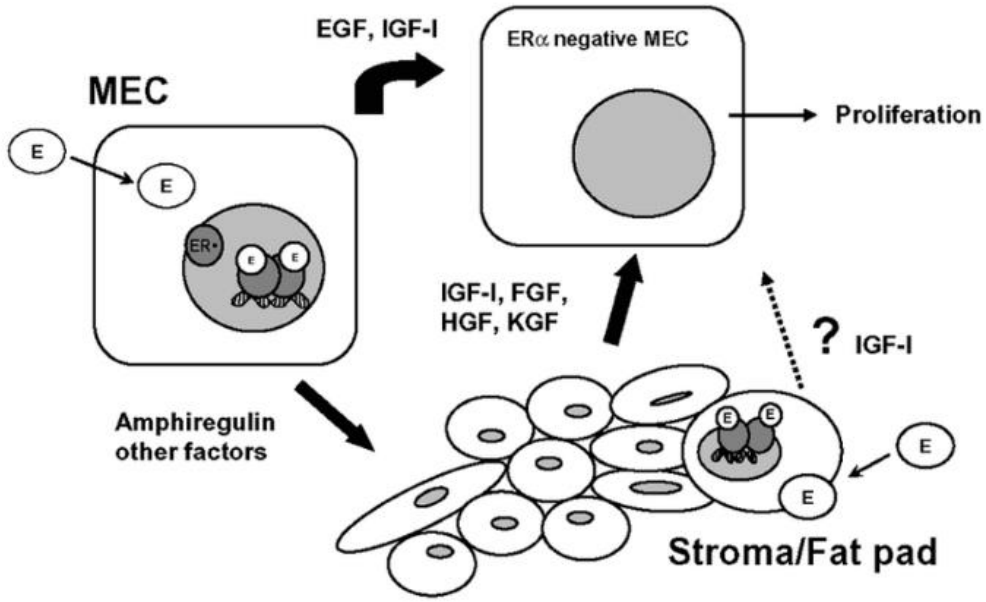

Figure 1.4: Estradiol pathway. Estradiol is thought to induce mammary growth by causing release of AREG, which binds EGF receptors on stromal cells, inducing FGF release. FGFs then induce proliferation of epithelial cells, which express fibroblast growth factor receptor 2. Figure from Connor et al. 2007.

1.2.2 Progesterone

P_4 is a paracrine steroid hormone similar in structure to E_2 and mainly produced in the ovaries (Macias et al. 2012). However, it is not required at the same periods as E_2 for mammary gland development, as PR knockout does not change the mammary phenotype during puberty (Lydon et al. 1995). On the other hand, signaling via PR is required for tertiary branching and alveogenesis (Lydon et al. 1995; Brisken et al. 1998). PRB is expected to be the dominant form in mammary gland development, though PRA may also be involved, including via its interaction with PRB (Mulac-Jericevic et al. 2003). Action of P_4 is also synergistic with PRL (Macias et al. 2012). P_4 is thus essential to mammary gland development, but so is its receptor PR. P_4 activates

transcription of receptor activator of NFKB1 ligand (RANKL) which through its receptor activates another distinct set of proliferation-associated genes (Figure 1.5) (Fernandez-Valdivia et al. 2008). Involvement of RANK is evidenced through mice with a RANKL or RANK knockout, which fail to undergo proper formation of alveoli, similarly to mice harbouring a PR knockout (Fata et al. 2000). P_4 also stimulates epithelial expression of Wingless/Integrated (Wnt) pathway members, such as Wnt4, another factor involved in branching morphogenesis (Brisken et al. 2000).

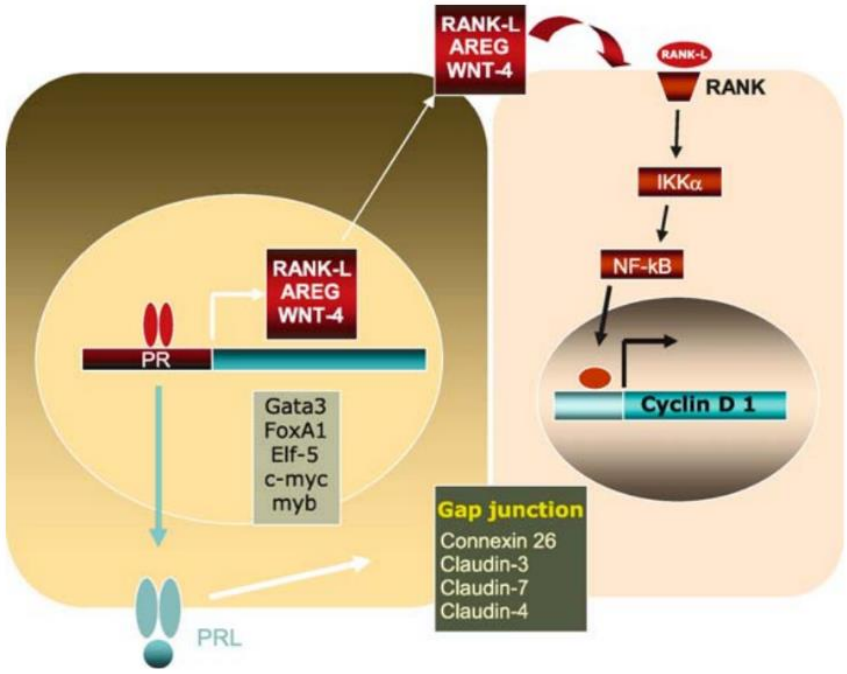

Figure 1.5: Progesterone pathway. Progesterone and its receptor are essential to tertiary branching and alveogenesis, among other mammary gland processes. Downstream effectors of their pathway include RANK and RANKL, as well as the β-catenin pathway. The canonical pathway is shown; other pathways activated by PRA/PRB interaction and membrane PR (PGRMC1) exist, adding complexity to progesterone signaling. Figure from Conneely et al. 2007.

1.2.3 Prolactin

PRL is a peptide hormone secreted by the anterior pituitary and regulated by the hypothalamus, but also produced in the mammary gland (Macias et al. 2012; Freeman et al. 2000). During pregnancy, PRL contributes to generation of alveoli, and during lactation its pathway conserves the complex structure of the mammary gland (Macias et al. 2012). This occurs via action on epithelial cells and indirectly by cross-regulation of P_4 (Ormandy et al. 1997). Its pathway is activated by nipple stimulation at lactation, which causes sensory nerves linked with the spinal

cord to transmit impulses to the hypothalamus, inhibiting the release of dopamine, which then removes its suppressive effects on PRL (Al-Chalabi et al. 2022). In parallel, OXT production leads to milk ejection from luminal cells, suppressing the dopamine pathway, further increasing PRL (Al-Chalabi et al. 2022). PRL transduces stimuli from the extracellular matrix during lactation via its interplay with integrin through the membrane protein signal regulatory protein alpha (SIRPA), which when activated, generates a complex with Jak2, resulting in activation of signal transducer and activator of transcription 5 (STAT5) (Galbaugh et al. 2010). This results in increased milk component synthesis, such as the proteins casein and whey acidic protein (WAP) (Yamaji et al. 2013). PRL activity is removed by suppressor of cytokine signaling 1 (SOCS1), which blocks activation of STAT5 via Jak2 (Lindeman et al. 2001) (Figure 1.6).

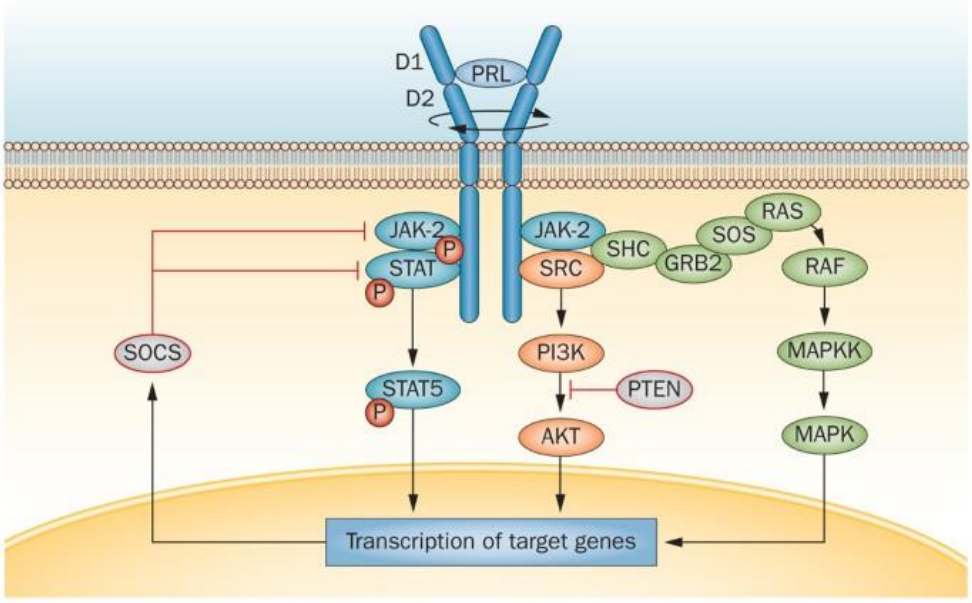

Figure 1.6: Prolactin pathway. Activation of members of the prolactin pathway results in alveogenesis and milk component synthesis, and includes cross-talk with progesterone, oxytocin and domapine. Mediators include Jak2 and STAT5, among others. Figure from Bernard et al. 2015.

1.2.4 Oxytocin

OXT is a peptide hormone most active at lactation. Its secretion is triggered by offspring suckling, which causes nerve impulse transmission to the hypothalamus, causing OXT secretion from the posterior pituitary and binding to receptors (OXTR) on myoepithelial cells, illiciting their contraction (Gimpl et al. 2001; Lollivier et al. 2006). The cellular effects most likely occur through the IP_3/Ca^{2+} signal transduction pathway (Nakano et al. 2001; Gàrriz et al. 2021).

1.3 Regulation of mammary gland development by Cx43

In addition to hormones, development of the mammary gland is mediated and enabled by gap junctions (Stewart et al. 2015; El-Sabban et al. 2003). Gap junction intercellular communication (GJIC) is a critical process, as it allows direct molecular communication between cells, coordinating, among others, growth, division and differentiation (Goodenough et al. 2009; Shaw et al. 2007; Zefferino et al. 2019; Delmar et al. 2018).

1.3.1 Gap junction definition and structure

Gap junctions are formed by bidirectional channels comprised of connexin transmembrane proteins (Stewart et al. 2015). Connexins are transcribed from various genes including GJA1 for connexin 43 (Cx43). These proteins arrange in hexamers, forming the connexon hemichannel (Laird 2006). Hemichannels from opposing membranes assemble to form channels, which concentrate in gap junction plaques, also termed mature gap junctions (Laird 2006). Individual connexins contain four hydrophobic, α-helical transmembrane domains connected by three loops, two of which protrude from the extracellular face of the membrane, and one from the cytosolic face (Delmar et al. 2018). They also contain the cytoplasmic N and C-terminal domains, subject to post-translational modifications and interactions affecting gating properties (Kirichenko et al. 2021).

1.3.2 Gap junction function

Gap junctions are present in almost all tissues, but they differ depending on the tissue type, as the connexins expressed and constituting gap junctions are different in different tissues (Goodenough et al. 2009). These vital structures allow direct transfer of small molecules approximately under 1 kilodalton (kDa) between adjacent cells (Stewart et al. 2015). For Cx43, most studies agree that it is expressed in myoepithelial gap junctions (Stewart et al. 2015); however, there is also evidence of Cx43 expression in luminal cells (Monaghan et al. 1996). Interestingly, gap junctions can be heterotypic, where the two opposing cells contain different hemichannels, and/or heteromeric, where the individual connexins within one hemichannel are different. In other words, gap junctions need not be comprised of only one connexin type (ie. homomeric and homotypic). In the mammary gland, gap junctions are present between luminal cells, between myoepithelial cells and between fibroblasts in the stroma. Evidence also suggests potential inter-cell type (ie. luminal to myoepithelial) gap junctions (Talhouk et al. 2005), including

some with presence of Cx43 (Dianati et al. 2016). By enabling molecular communication between cells of the mammary gland, GJIC is indispensable to its complex, space and time-dependent development and function (Stewart et al. 2015). Table 1 presents molecule types and examples for each type, that are permeable to gap junctions or connexin hemichannels. For each molecule type, it is possible that additional species are permeable, but research has not validated all molecules of the given categories.

Table 1.1: Examples of molecule types permeable to gap junctions

Molecule Type	Examples of molecules	Studies
Carbohydrates	Sucrose, mannitol, lactate, pyruvate	Locke et al. 2004; Wang et al. 1997; Lounas et al. 2024
Nucleotides	ATP, ADP, AMP	Goldberg et al. 2002
Nucleic acids	siRNA, miRNA	Valiunas et al. 2005, Zong et al. 2016
Second messengers	IP_3, cAMP, cGMP, Ca^{2+}, Mg^{2+}	Bedner et al. 2006; Hernandez et al. 2007; Sáez et al. 1989
Amino acids	Glutamate	Takeuchi et al. 2006
Solvents	Water	Chipman et al. 2003

1.3.3 Cx43 expression is stage-specific; correlated with hormone expression

There exist 20 murine and 21 human genes coding for the connexin family, members of which are named after their molecular weight (Söhl et al. 2004). Of those members, four are detected in the mouse mammary gland (Cx43, Cx26, Cx30 and Cx32) (Dianati et al. 2016). Cx43 and Cx26 are the main Cxs the human breast, but there is evidence that others are detected as well (Monaghan et al. 1996; Teleki et al. 2014). Expression of the four mouse mammary gland Cxs across development has been tested previously by Western blot and RT-qPCR (Dianati et al. 2016). Briefly, Cx43 expression remains elevated throughout the stages, and reaches a well-defined valley from lactation day seven to fourteen. Cx26 and Cx30 remained low before week 10 (adulthood), but reach peaks at pregnancy day 18, after which baseline levels slowly returned at involution. Last, Cx32 also had overall low expression, but peaked at lactation day seven. The peaks and valleys of Cxs' expression at various time points suggest that they play important stage-specific functions (Stewart et al. 2015). Their expression also correlates with mammary gland hormone expression; for example, lactation is characterized by elevated PRL and OXT and lower E_2 and P_4 levels, whereas Cx43 is minimally expressed (Dianati et al. 2016). It is thus anticipated that regulation of Cxs may be linked with pathways activated or deactivated by various hormones across development. This project focuses on Cx43 because its role in mammary gland

biology is crucial, and it can contribute to the development of breast cancer (Chasampalioti et al. 2018; Busby et al. 2018).

1.3.4 Cx43 Gene Structure

Cx43 is located on chromosome 10 in the mouse (Iacobas et al. 2007) and six in the human (Oyamada et al. 2013). In the mouse, Cx43 is encoded by six exons, with five 5' UTRs and one coding region (Figure 1.7) (Oyamada et al. 2013). Upon transcription, Cx43 mRNA is subject to alternate splicing, resulting in nine different mature mRNA molecules discovered for this gene in mice (Pfeifer et al. 2004). Some of those different mRNA can also arise from use of three alternate GJA1 promoters (Oyamada et al. 2013).

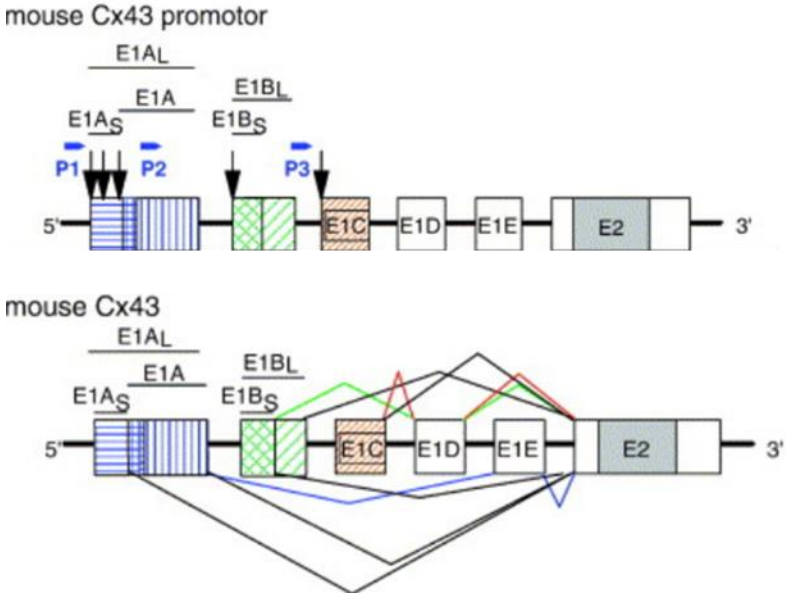

Figure 1.7: GJA1 gene structure. The gene encoding Cx43, GJA1, contains three alternative promoters used in different conditions and resulting in different mRNA transcripts. Also affecting mature Cx43 mRNA are alternate splicing patterns. The letters denote exons (E) and promoter (P) regions. Figure from Oyamada et al. 2005.

1.3.5 Lifecycle of Cx43

As a transmembrane protein, Cx43 is synthesized and transported through the endomembrane system, which includes translation by endoplasmic reticulum-bound ribosomes into its membrane (Figure 1.8) (Dbouk et al. 2009). It is then transferred by vesicles to the golgi apparatus, during which hexamerization begins and lasts until export from the trans-golgi network, resulting in

connexon hemichannels (Musil et al. 1993; Laird 2006; Smyth et al. 2012). This is interestingly in contrast to other connexins which are thought to oligomerize inside the endoplasmic reticulum (Laird 2006). Connexons are targeted to the membrane after transfer through the trans-golgi network (Smyth et al. 2012). Two connexons from opposing membranes dock, forming a bidirectional channel, and the final gap junction consists of concentrated regions of bidirectional channels assembled into plaques (Contreras et al. 2003). Connexons can also act as hemichannels, though not in all tissue types and connexin isoforms (Contreras et al. 2003). Endoplasmic reticulum-associated degradation via the proteasome, which degrades and recycles misfolded proteins, may result in Cx43 degradation prior to exit from this organelle (Aasen et al. 2019). Another degradation route, connexosomes, is characterized by an internalized double-membrane structure with parts of gap junctions that ends up fusing with the lysosome (Laird 2006). Post-translational modifications occur at all steps after Cx43 synthesis, affecting individual connexins, their hemichannels and functional gap junctions (Puranam et al. 1993; Johnstone et al. 2012).

1.3.6 Loss of Cx43 – what is the effect?

Mice lacking Cx43 die rapidly due to improper electrical coupling in heart cells. In an older article, embryonic stem cells with a heterozygous mutation of GJA1 were injected into C57BL/6 blastocysts, generating wild type, $GJA1^{+/-}$ and $GJA1^{-/-}$ mice; none of the homozygous mutated mice survived for greater than one day due to cardiac malformations (Reaume et al. 1995). Mice with heart-restricted knockout of GJA1 survived past birth, but death occurred after several weeks due to ventricular arrhythmia (Gutstein et al. 2013). In the mammary gland, it has been found that mice expressing mutated Cx43 display decreased GJIC (Plante et al. 2008). The epithelia of their mammary glands were characterized by delayed development and inability to undergo milk secretion. Thus, lack of Cx43 results in developmental delay and loss of function of the mammary gland.

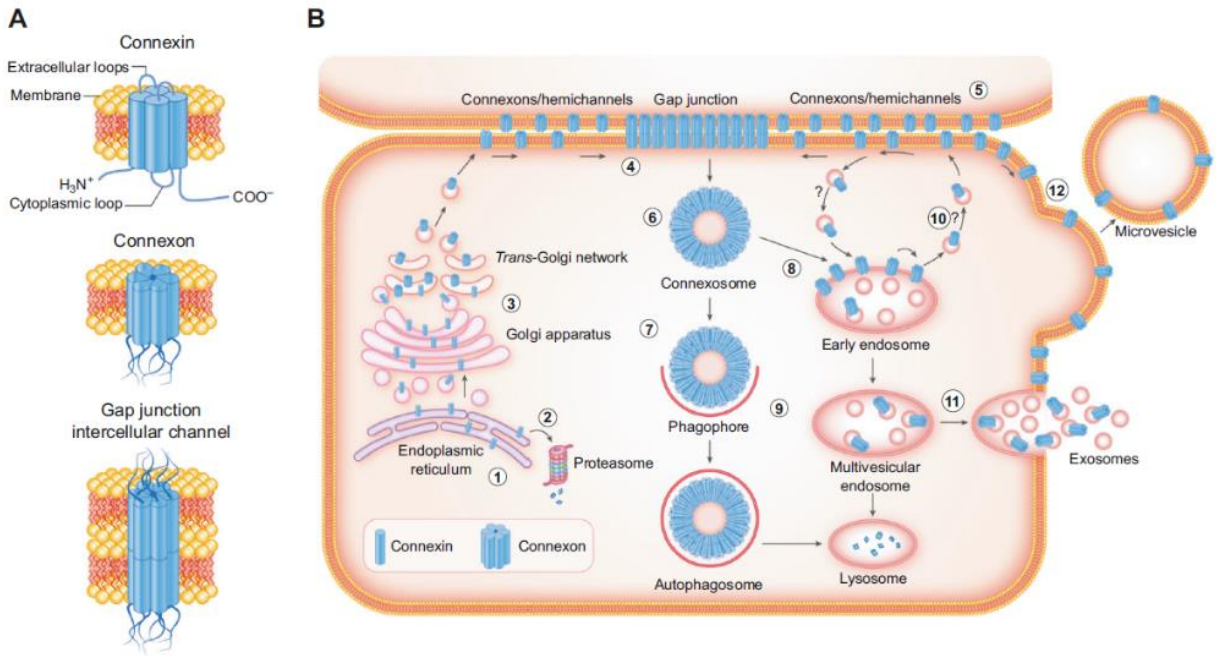

Figure 1.8: Lifecycle of Connexins. Connexins' turnover is rapid, with synthesis beginning at endoplasmic reticulum-bound ribosomes and followed by post-translational modifications and targeting to the membrane. The proteasome, connexosome and endosome are three alternate routes of Cx43 degradation affecting its steady-state abundance. Figure from Aasen et al. 2019.

1.4 Known pathways and factors in the regulation of Cx43

It has been demonstrated that Cx43 has a short turnover of a few hours (Epifantseva et al. 2018). Levels of Cx43, as well as GJIC, can be rapidly increased or decreased according to the needs of the tissue (Laird et al. 2006). For instance, a strongly increased Cx43 expression has been observed in the uterus of mice upon onset of partuition, likely allowing synchronised contraction of uterus muscles during delivery (Tong et al. 2009). Rapid expression of Cx43 allows GJIC between antigen-presenting cells, leading to activation of other immune cells and a strengthened immune response (Xu et al. 2019). In the nervous system, Cx43 regulates permeability of the blood-brain barrier, as well as the dynamic process of behaviour, learning and memory (Dong et al. 2022). These studies suggest the existence of various mechanisms of regulation allowing short-term regulation of Cx43 within tissues. Indeed, it has been demonstrated that Cx43 can be regulated at both transcriptional and post-transcriptional levels.

1.4.1 Histone Modifications

Acetylation of histones is a type of modification that relieves chromatin condensation and enables interacting proteins to engage with uncovered DNA regions, triggering transcription of genes and subsequent biological processes (Unnikrishnan et al. 2010). In rat liver transformed epithelial cells, exposure to the histone deactylase inhibitors suberoylanilide hydroxamic acid and trichostatin-A increased Cx43 mRNA and protein levels (Ogawa et al. 2005). In human peritoneal mesothelial cells, the same authors demonstrated increased acetylation of GJA1-linked histones H3 and H4 using chromatin immunoprecipitation assays, confirming the specificity of suberoylanilide hydroxamic acid and histone deacetylases to GJA1. In prostate cancer cells, trichostatin-A was shown to synergize with a coactivator to activate GJA1 transcription in an acetylation-dependent mechanism (Hernandez et al. 2006). Lastly, one study showed that transfection of a Cx43 plasmid in conjunction with exposure to the histone deacetylase inhibitor 4-phenylbutyrate, greatly enhanced Cx43 expression and resulted in reduced growth of human nasopharyngeal tumour cells (Hattori et al. 2007). These studies show that histone condensation state and acetylation are factors in Cx43 expression levels (Figure 1.9).

1.4.2 Transcription Factors

Specificity protein 1 (SP-1) is a transcription factor that binds GC-concentrated regions of DNA using its three zinc-finger domains (Kaczynski et al. 2003). On the other hand, activator protein 1 (AP-1) is a heterodimeric transcription factor formed by members of the JUN and FOS family that via basic leucine zipper (bZIP) domains bind to AP-1 consensus sequences of DNA (Atsaves et al. 2019; Ransone et al. 1993). Here, the basic region of the bZIP domain mediates interactions with the consensus sequence, whereas its leucine zipper region mediates dimerization of the two transcription factors (Wen et al. 2021). One study using *Drosophila* SL2 cells and an electrophoretic mobility shift assay demonstrated that SP-1 and SP-3 bind to four predicted binding sites in the Cx43 gene, affecting its promoter activity (Figure 1.9) (Teunissen et al. 2003). Using a chromatin immunoprecipitation assay, it was demonstrated that SP-1 and AP-1 activation of the GJA1 promoter was deficient in metastatic melanoma cells with reduced protease-activated receptor 1 (PAR-1) expression, suggesting that normal Cx43 expression depends on the transcription factors SP-1 and AP-1, but may also necessitate PAR-1 (Villares et al. 2009). Interestingly, trichostatin-A activation of transcription via its inhibition of histone deacetylase

activity was linked with SP-1 and AP-1 access to their binding regions (Hernandez et al. 2006), connecting histone acetylation and transcription factors as mechanisms of Cx43 regulation.

Cyclic AMP (cAMP) activates GJIC through enhancement of Cx43 transcription and changes in its localization (Yu et al. 1994; Oyamada et al. 2013). GJA1 promoter P1 is responsive to cAMP, shown by a luciferase assay (Civitelli et al. 1998), but strangely, activation has not been proven to depend on the cAMP-responsive element (de Montgolfier et al. 2011). The Wnt/β-catenin pathway also contributes to GJA1 expression. PC12 cells, which are derived from a rat adrenal medulla pheochromocytoma, displayed increased Cx43 mRNA and protein upon overexpression of Wnt1, while other Cxs were left unaffected (van der Heyden et al. 1998). In addition, in osteocytes, direct binding of the Cx43 promoter by β-catenin resulted in increased Cx43 expression and GJIC (Xia et al. 2010). Changes in GJA1 gene transcription by factors not occurring in all cell types (ie. not ubiquitous), have also been demonstrated for Nxk2.5, Shox2, Irx3, Tbx2, Tbx3 and Tbx18 (Oyamada et al. 2013). Further evidence is needed as Cx43 regulation by transcription factors in the mammary gland has not been studied deeply.

1.4.3 DNA Methylation

Different studies show that DNA methylation is an important factor in Cx43 expression and cancer progression (Figure 1.9). A qPCR-based methylation experiment showed that in lung cancer, decreased Cx43 expression commonly displayed in tumour cells can be due to excessive promoter methylation (Chen et al. 2003). Further, the electrophoretic mobility shift assay of these authors demonstrated that interestingly, methylation disables AP-1 binding to the GJA1 promoter in contrast to normal tissues. Cx43 expression was also measured in HEK293T cells, which are characterized by normal Cx43 expression, and HeLa cells, harbouring reduced Cx43 expression, treated with and without the methyltransferase inhibitor 5-Aza-CdR. Upon 5-Aza-Cdr exposure, HeLa cells had increased gap junctions, similar to Cx43 positive HEK293T cells (Kong et al. 2020). Lastly, decreased Cx43 levels characteristic of glioma stem cells were found to be due to excessive promoter methylation, shown using immunostaining and qPCR assays (Yu et al. 2012). These studies indicate that DNA methylation catalyzed by various enzymes is a mechanism of Cx43 regulation, accounting for its suppression in various cells and tissues (Dhar et al. 2021).

1.4.4 Alternative splicing

Alternate splicing patterns and promoter activity result in Cx43 isoforms ranging from 7 kDa to the full-length protein product (Smyth et al. 2013). Interestingly, 20 kDa Cx43 is the most expressed of the truncated forms, interacting with standard Cx43 and necessary to its trafficking to the membrane (Figure 1.9) (Shimura et al. 2022). This process is likely related to the mTOR pathway, as its inhibition in different tissues and cells resulted in increased internal translation of Cx43, and higher expression of the 20 kDa isoform (Smyth et al. 2013; Basheer et al. 2016; Salat-Canela et al. 2014).

1.4.5 Phosphorylation

Phosphorylation is one of the most widely studied and impactful post-translational modifications of Cx43, regulating its stability at the plasma membrane, hexamer formation and disassembly, degradation, channel permeability and protein-protein interactions (Table 1.2, Figure 1.9) (Lampe et al. 2004; Solan et al. 2020). In addition, phosphorylation by kinases acting on Cx43 such as PKA, PKC and MAPK, among others (Lampe et al. 2004), can occur at many different sites, with examples shown in Table 2 below. Breast cancer development is associated with Cx43 expression level and localization, which are processes affected by phosphorylation; in this way, phosphorylation is also an attribute contributing to the disease (Zhang et al. 2023).

Table 1.2: Examples of Cx43 Phosphorylation Sites and their Effect on GJIC.

Phosphorylation site	Enzyme involved	Impact on GJIC	Study
Ser368, Ser372	Phosphorylation by PKC	GJ opening	Akoyev et al. 2007; Yang et al. 2019
Ser262, Ser368	Phosphorylation by tissue peptide antigen (TPA)	Decrease in intercellular communication (site 368)	Akoyev et al. 2007
Ser255	Phosphorylation by mitogen-activated protein kinase (MAPK)	GJ internalization, GJ closure	Nimlamool et al. 2015
Tyr274, Tyr265	Phosphorylation by C-Src	GJ closure	Zhang et al. 2022
Ser325, Ser328, Ser330	Phosphorylation by casein kinase 1 (CK1)	Formation of GJ plaques	Cooper et al. 2002
Tyr247, Tyr265	Phosphorylation by V-Src and C-Src	GJ closure	Solan et al. 2008

Ser364, Ser365	Phosphorylation by PKA	Membrane trafficking	Shah et al. 2002; TenBroek et al. 2001; Solan et al. 2007
Ser373, Ser369	Phosphorylation by Akt and PKA	Membrane trafficking, interaction with ZO1 and 14-3-3 (Site 373)	Park et al. 2006; Park et al. 2007
Ser368	Phosphorylation by PKC	GJ closure	Lampe et al. 2000; Solan et al. 2007
Ser279, Ser282	Phosphorylation by mitogen-activated protein kinases (MAPKs)	GJ closure	Leykauf et al. 2003; Petrich et al. 2002; Polontchouk et al. 2002
Ser255 and Ser262	Phosphorylation by MAPKs and cyclin dependent kinase 1 (CDK1)	GJ closure	Sirnes et al. 2009; Kanemitsu et al. 1998; Lampe et al. 1998

GJ = Gap junction.

1.4.6 SUMOylation

SUMOylation refers to covalent addition of small ubiquitin-like modifier groups to lysine residues by the enzymes SUMO E1, E2 and E3, and removed by SUMO proteases (Maejuma et al. 2014). This modification regulates several core cellular processes, including post-translational processing of proteins such as Cx43 (Figure 1.9) (Yang et al. 2017; Aasen et al. 2019). Mutation of key lysine residues of Cx43 has been shown to decrease its SUMOylated fraction, decreasing expression of Cx43 and the number of active gap junctions (Kjenseth et al. 2012). In another example, a recent study on rat hearts found that decreased levels of protein inhibitor of activated STATy (PIASy), a SUMO E3 enzyme, resulted in decreased SUMOylation of Cx43 (Wang et al. 2023). Another study identified that Cx43 is less SUMOylated in liver cancer stem cells than control cell lines, and mutation of key lysine residues resulted in decreased SUMO1 interaction with Cx43 (Shen et al. 2018). In addition, co-expression of Cx43 and SUMO1 had the effect of increasing GJIC in the select cell line (Shen et al. 2018). Last, Zhang et al. 2018 found that by silencing Ubc9, a SUMO E2 enzyme, SUMOylated Cx43 decreased, and less Cx43 was localized in the membrane.

1.4.7 Ubiquitination

Ubiquitination is the covalent addition of ubiquitin to proteins, also catalyzed by activating (E1), conjugating (E2) and ligase (E3) ubiquitin enzymes (Guo et al. 2023). Mono-ubiquitination alters

diverse protein properties such as sorting, trafficking, stability, localization, interactions or activity (Figure 1.9) (Hicke et al. 2003; Sewduth et al. 2020). Polyubiquitination stemming from lysine 48 and 63 of their subunits targets proteins for 26S proteasome-mediated degradation and endocytosis respectively (Guo et al. 2023). Indeed, polyubiquitination of Cx43 and its likely degradation via the proteasome was demonstrated in an older article (Laing et al. 1995). TS20 cells contain a heat-sensitive E1 ubiquitin enzyme, which, when deactivated, cannot participate in the ubiquitination and degradation of Cx43 (Laing et al. 1995). The authors showed that TS20 cells when treated with heat, had unaffected rather than decreased Cx43 levels, demonstrating the importance of ubiquitination in Cx43 expression. Polyubiquitination was also confirmed in this study using immunoprecipitation, where a large size range of Cx43-ubiquitin complexes were detected.

1.4.8 Protein-protein interactions

Gap junctions are associated with drebrin, which connects them to actin, as well as EB1, which connects them to microtubules, linking Cxs with filaments of the cytoskeleton (Shaw et al. 2007; Butkevich et al. 2004). In this way, Cx43 is able to regulate not only intercellular communication, but also cytoskeletal polymerization and depolymerization cycles leading to cell movement and/or attachment to each other (Francis et al. 2011). Actin is also necessary for proper localization of gap junctions (Epifantseva et al. 2018). Cx43 interacts with other junctional complexes, such as tight junctions, which create cell polarity by restricting molecule passage, and adherens junctions, which link cells to each other and their cytoskeletons (Dianati et al. 2016). For example, β-catenin (adherens junctions), E-cadherin (adherens junctions) and claudin 7 (tight junctions) have been shown using co-immunoprecipitation methods to interact with Cx43, forming a junctional nexus (Dianati et al. 2016). Stage-specific interactions between many components of the nexus (Dianati et al. 2016) could suggest potential stage-specific variation in miRNA expression, and possibly multiple junctional protein regulation by single miRNAs.

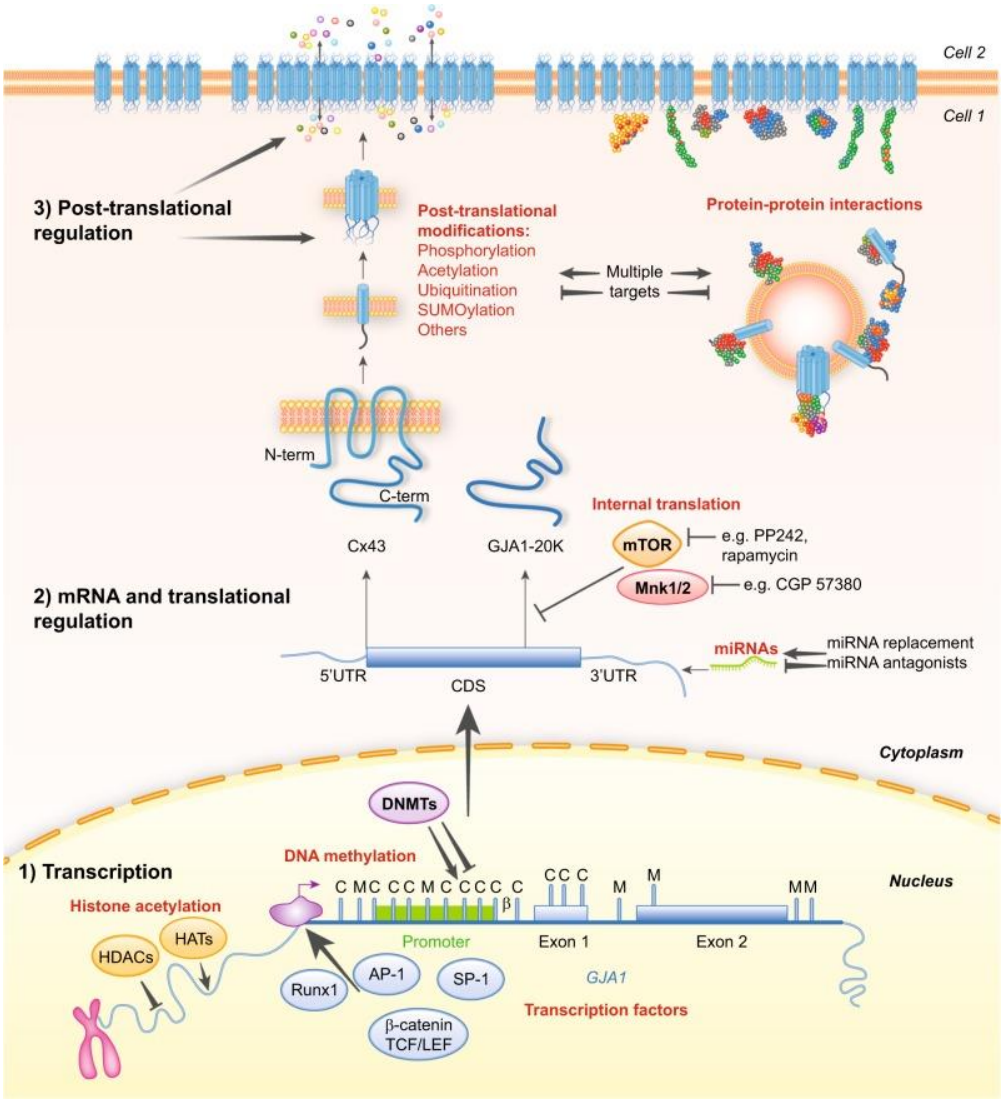

Figure 1.9: Mechanisms of Cx43 Regulation. The gene coding for Cx43, GJA1, is subject to histone (de)acetylation, methylation and interactions with transcription factors, affecting its level of transcription. Protein Cx43 is regulated by phosphorylation, ubiquitination, SUMOylation, acetylation, as well as protein-protein interactions. Figure from Aasen et al. 2019.

1.4.9 Effect of hormones on Cx43 expression

There are some experiments demonstrating that Cx43 is likely regulated by or associated with circulating hormones, but information is scarce. Cx43 is known to be necessary for spermatogenesis (Roscoe et al. 2001), and in the brook trout testis, cAMP and triiodothyronine were found to regulate GJA1 by interacting with its promoter (de Montgolfier et al. 2009; de Montgolfier, et al. 2011). However, not much is known on the effect of hormones on Cx43 expression in the mammary gland. In studying the biphasic ovalatory cycle, one group found that

endometrial stromal cells treated with a combination of E_2, P_4 and cAMP (E/P/c) displayed positively regulated differentiation as seen in differential expression of various markers (Yu et al. 2016). This process was associated with increased mRNA and protein levels of Cx43 especially after three days of exposure, and Cx43 increase was reversed upon hormone withdrawal at three and seven days (Yu et al. 2016). The E/P/c treatment was also associated with increased PRL secretion in the media, as well as characteristics linked with epithelial-mesenchymal transition.

Treatment with E_2 increases Cx43 expression in MLO-Y4 osteocyte-like cells (Ren et al. 2013). The authors also found that ER knockdown by siRNA and by the antagonist fulvestrant, each caused decreases of Cx43 levels. This is important, as GJIC in osteocytes contributes to transduction of mechanical stimuli, such as physical stress, leading to bone loss or formation (Ren et al. 2013). Interestingly, in one study, ER inhibition was also linked with increased Cx43 ubiquitination, which lead to its decreased levels (Tsai et al. 2018). Both studies show that Cx43 expression is dependent on E_2 action in the select cell lines. Cx43 expression is also sensitive to E_2 in uterine endometrial stromal cells; upon exposure to a 100 nM concentration, a more than 8-fold increase was observed (Liu et al. 2019). The same study found upregulation of PR by over 2-fold with 100 nM treatment of E_2.

In the American mink, PRL was measured by immunoblot as maximal at lactation, consistent with reviews on the elevated role of prolactin at this stage (Vitale 2001). PRL was associated with an increase in Cx43; however, measurements were taken from the anterior pituitary and not mammary gland itself. The results suggest that Cx43 is linked with PRL expression during the reproductive cycle and is involved in its coordination. Due to conflicting results about the role of connexins in breast development, authors have sought out to clarify the role of Cx26 and Cx43 in lactation (Mroue et al. 2015). In their study, Cx26-overexpressing mice failed to undergo milk ejection despite normal milk production, and in myoepithelial cells, overexpression of Cx26 resulted in decreased Cx43, suggesting that Cx26 could affect Cx43 and mammary gland function. This lead them to use an *ex-vivo* organoid model, demonstrating that knockdown of Cx43 by short haipin RNA resulted in loss of contraction ability upon OXT administration. Thus, Cx26, Cx43 and contraction are at least partially linked with the hormone OXT. Overall, Cx43 regulation by hormones is crucial, but excessive research gaps exist that make these phenomena difficult to fully understand.

1.5 Regulation of Cx43 by miRNA

In the last few years, an increasing amount of studies have demonstrated that Cxs' expression can be regulated by miRNA in various tissues, but still little information is available regarding the role of miRNA in their regulation during mammary gland development, and how hormones contribute to this regulation. Examples of Cx43 regulation by miRNA in various tissues and organs is presented in Table 3, below.

1.5.1 miRNA and Cx43 expression

The first miRNA was identified approximately 30 years ago (Lee et al. 1993). pri-miRNA are transcribed by RNA polymerase II or III in the nucleus (Lee et al. 2004; Borchert et al. 2006), are processed into pre-miRNA (Denli et al. 2004) and are then exported into the cytosol (Figure 1.10) (Okada et al. 2009). There, they are processed further and loaded into argonaute (AGO), forming the RNA-induced silencing complex (RISC) (Yoda et al. 2010). This complex binds to the translation start site of mRNA, blocking translation, or 3' UTR, causing transcipt cleavage and degradation, based on the degree of complementarity between miRNA and target mRNA region (Jo et al. 2015). The final miRNA product can range from 19 to 25 nucleotides (Reichholf et al. 2019). Interestingly, miRNA transcribed from distinct genes, also known as canonical miRNA, or miRNA generated by non-canonical mechanisms, the most common of which is miRNA produced from intronic regions of RNA (intronic miRNA, or mirtrons), have been identified (Reichholf et al. 2019).

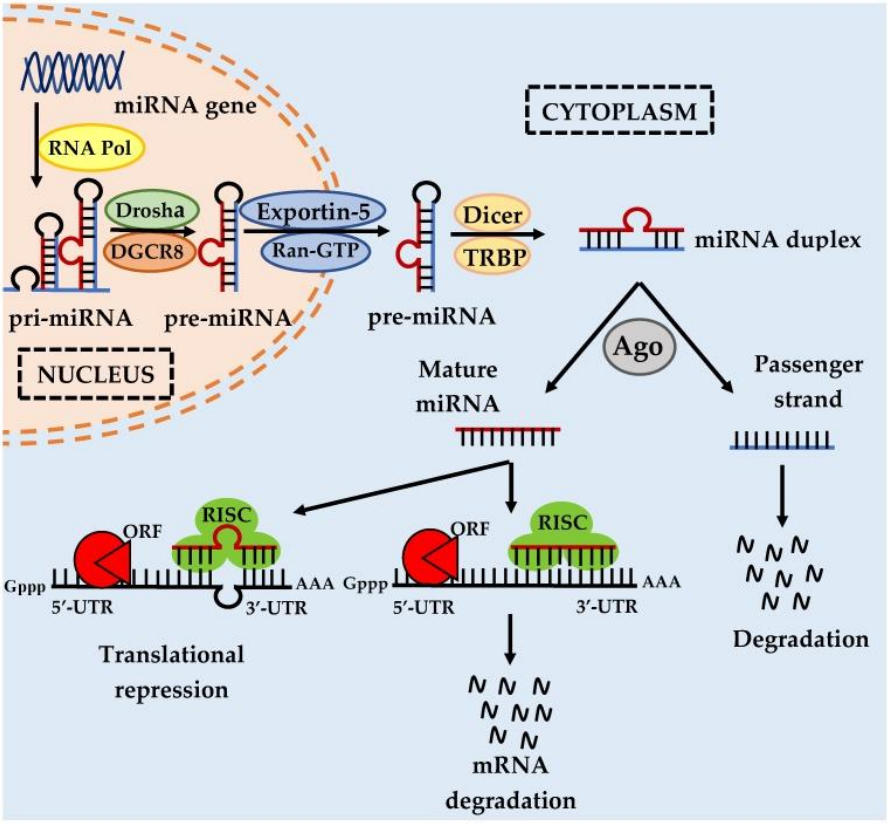

Figure 1.10: Biogenesis of MicroRNA. Primary miRNA transcripts are synthesized in the nucleus and are processed into pre-miRNA. Pre-miRNA species are then exported into the cytoplasm, where further processing and loading into the RISC complex occur. The two main mechanisms of gene expression regulation are blockage of translation or mRNA cleavage. Figure from Loh et al. 2019.

Table 1.3: Examples of Regulation of Cx43 Expression by MicroRNA in Various Cell Lines, Tissues and Organs.

MicroRNA	Tissue or cell line	Findings	Reference
miR-206	Transgenic mice overexpressing miR-206; HL-1 cells	miR-206 overexpression in transgenic mice resulted in inhibited Cx43 expression in the atria and ventricles, which lead to cardiac arrhythmias. The findings were confirmed using a luciferase assay *in vitro*, showing that miR-206 targeted Cx43 via the 3' UTR of its mRNA.	Jin et al. 2019
	Primary bone mesenchymal stem cells	Treatment with the flavonoid antioxidant quercetin increased bone mineralization and expression of osteoblast-specific genes by decreasing miR-206 and increasing Cx43 expression. miR-206	Zhang et al. 2020

		overexpression abolished the effects of quercetin on bone generation, in addition to suppressing Cx43 expression via the 3' UTR of its mRNA.	
	Primary mouse osteoblasts, mice overexpressing miR-206	Targeting of Cx43 mRNA 3' UTR by miR-206 was identified as an important regulation axis affecting differentiation during skeletal muscle development. Mice overexpressing miR-206 displayed impaired bone formation, indicating the importance of this axis in differentiation.	Inose et al. 2009
	Mouse model of sepsis-induced acute lung injury, generated using caecum ligation and perforation	Cx43 was linked with alveolar cell permeability, and miR-206 overexpression resulted in reversed phenotypes of sepsis-induced acute lung injury. In ATII cells, miR-206 directly targeted Cx43 via the 3' UTR of its mRNA.	Zhou et al. 2019
	Epithelial ovarian cancer cells	The miR-206 overexpression group had increased tumour size in the A2780 and not OV2008 cell injection, but upon treatment with cisplatin, both A2780 and OV2008, miR-206 overexpression groups displayed increased tumour size relative to controls. In other words, prior to cisplatin administration, miR-206 level was a factor of tumour progression in one cell line, but when cisplatin was administered, miR-206 became a factor of progression in both cell lines by conferring cisplatin chemoresistance to epithelial ovarian cancer cells.	Yu et al. 2020
miR-206, miR-1	HeLa cells; C2C12 cells	Upon addition of low serum media, C2C12 cells had increased myogenin, a muscle differentiation marker, as well as increased miR-206 and decreased Cx43, as tested using northern blot and immunofluorescence experiments. Transfection of miR-1 and miR-206 inhibitors resulted in elevated Cx43 levels compared with controls, characteristic of undifferentiated C2C12 cells, confirming the involvement of this axis in development. miR-1 and especially miR-206 target Cx43 directly via the 3' UTR of its mRNA (luciferase assay).	Anderson et al. 2006
miR-1	Rat heart tissue, HEK293 cells	miR-1 suppresses Cx43 protein expression levels and its overexpression is associated with cardiac arrythmia.	Yang et al. 2007

miR-130a	Mice with tetracycline-inducible overexpression vector for miR-130a; 3T3 and HL1 cells	Mice possessed a 90% reduction of Cx43 after 10 weeks, which resulted in tachycardia and atrial and ventricular arrhythmias. Cx43 mRNA was directly targeted by miR-130a *in vitro*, as tested using a luciferase assay.	Osbourne et al. 2014
miR-221/222	U251 cells	miR-221/222 targeted Cx43 mRNA directly via its 3' UTR, and liposome transfection of a miR-221/222 inhibitor decreased invasion and proliferation and increased apoptosis. Thus, miR-221/222 partly contributes to glioma properties via regulation of Cx43.	Hao et al. 2012
miR-218	Nasopharyngeal carcinoma cells	Migration capacity was linked with miR-218, whose overexpression resulted in decreased Cx43 by qPCR. This miRNA directly targeted Cx43 mRNA via its 3' UTR via (luciferase assay).	Alajez et al. 2011
miR-20a	MDA-PCa-2b cells, mice	miR-20a inhibition in MDA-PCa-2b cells decreased their growth, and this miRNA was shown to inhibit Cx43 expression directly via the 3' UTR of its mRNA. Tumor volume was decreased in mice injected with MDA-PCa-2b cells stably depleted of miR-20a, relative to control mice.	Li et al. 2012a
miR-23b-3p, miR-342-3p	Flp-In T-Rex 293 cells	A crosslinking, ligation, and sequencing of hybrids (CLASH) experiment uncovered two interactors of Cx43 mRNA: hsa-miR-23b-3p and hsa-miR-342-3p.	Helwak et al. 2013
miR-206, 218-5p, 4266, 636, 648, 6888-5p, 595, 651-5p, 221-3p, 222-3p, 20a-5p, 342-3p, 23b-3p, 130a-3p	Various cell lines	Review of other literature that shows evidence of miRNA regulation of Cx43 expression.	Calderón et al. 2016

1.5.2 Progesterone, ZEB1/2, Cx43 and OXTR axis in the myometrium

As previously mentioned, information on hormone induction or suppression of Cx43, miRNA regulation of Cx43 and hormone effects on miRNA is scarce. However, there is one study that was able to link all three of these important processes (Renthal et al. 2010). In microarray

experiments, the above authors found that the miR-200/429 family expression in uterine tissue during late gestation was significantly upregulated, and their targets ZEB1 and ZEB2 were downregulated. It was also found that those transcriptional repressors (Spaderna et al. 2008) were able to silence Cx43 and OXTR expression, regulating transition to labour. Further, PR was found to induce ZEB1, suggesting a link between hormones, transcription factors, miRNA and Cx43. A key difference to address between this study and the mammary gland is that in the latter, Cx43 has been identified as lowest at lactation, which is the stage where myoepithelial cells must contract (Dianati et al. 2016). Overall, data suggest that the miR-200/429 family could be tightly integrated with mammary gland hormone expression and play a central role in its function via regulation of Cx43.

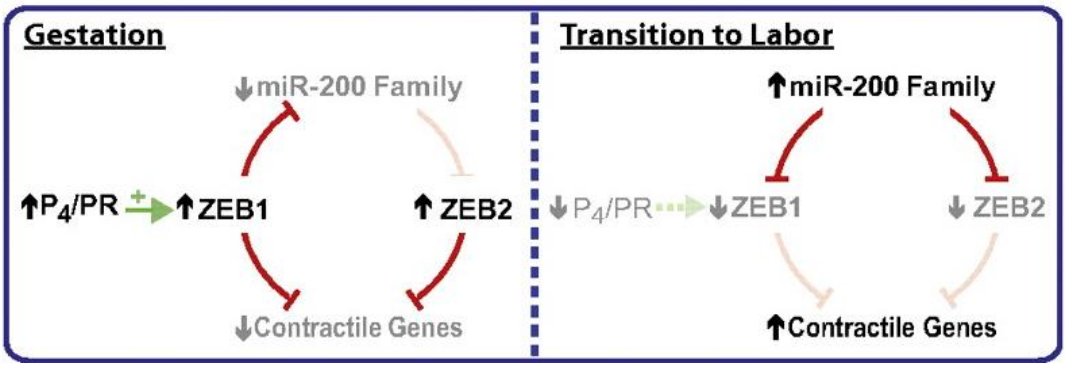

Figure 1.11: Uterine effects of progesterone on ZEB1/2, oxytocin receptor, Cx43 and other genes associated with labour (Renthal et al. 2010). Prior to labour, progesterone remains at high levels, keeping ZEB1/2 expression elevated and the miR-200/429 family and contractile genes suppressed. During labour, progesterone activity decreases, decreasing ZEB1/2 and relieving suppression of the miR-200/429 family and contractile genes. Figure from Renthal et al. 2010.

1.5.3 Regulation of Cx43 expression by miRNA in breast cells

In breast cancer cells, miR-1, miR-206, miR-200a, miR-381, miR-23a, miR-23b and miR-186 were identified as functional inhibitors of Cx43 expression using miRNA transfections, Western blot and RT-qPCR experiments (Ming et al. 2015a). Interestingly, luciferase assays showed that miR-200a-3p was able to target Cx43 directly at the 3' UTR of its mRNA, and was also linked with MCF7 cell migration (Ming et al. 2015a). In another study, increased miR-206 was associated with decreased migration and invasion of MDA-MB-231 and MDA-MB-436 cells by targeting Cx43 directly via the 3' UTR of its mRNA (Fu et al. 2015). Cx43 overexpression increased cell adhesion and GJIC *in vitro*, but miR-206 overexpression alone did not decrease tumour onset *in vivo* due

to dual functions of Cx43 in different stages of disease progression (Fu et al. 2015). C/EBPα, a transcription factor of Cx43, was identified as directly regulated by miR-381 in breast cancer cells, showing that miRNA can regulate Cx43 both directly and indirectly through its interactome (Ming et al. 2015b). Interestingly, it was also found recently that circRNAs, which have the ability to suppress miRNA expression, among other regulatory effects, might also be implicated in Cx43 regulation by miRNA (Kristensen et al. 2022). Lastly, in response to Cx43 knockdown, 65 miRNAs were identified as dysregulated, with 15 of those miRNA implicated in polarity of mammary epithelial cells (Nasar al Deen et al. 2021), which is important to morphogenesis of the mammary gland (Wang et al. 2011). It is important to note that his study discusses Cx43 regulation of miRNA, and not miRNA regulation of Cx43, but still shows that the two species are linked (Figure 1.11).

1.6 Regulation of miRNA by hormones

Hormones are important regulators of tissue development and function. Typically, hormones binding to their receptor will induce signaling pathways, resulting in the expression of numerous genes. Given the role of miRNA in gene regulation, it can be postulated that hormones will also regulate the expression of miRNA, thus contributing to the cellular and tissue response. A few studies have analyzed this relationship, including in the mammary gland and breast cancer cells.

1.6.1 Effect of estradiol on miRNA expression

Literature information of the role of E_2 in miRNA expression in breast cancer cells ranges from no effects to widespread impacts on the cellular miRNA profile. The effect of 10 nM E_2 treatment on both MCF-7 and ZR-75.1 breast cancer cells was assessed using microarray experiments (Ferraro et al. 2012). It was found that between the two cell lines, expression of 52 common miRNA were changed; dozens of others were altered in one out of the two cell lines. Interestingly, regulated miRNA included bioinformatics-predicted and *in vitro*-validated Cx43 regulators such as hsa-miR-125b (TargetScan Mouse 8.0, miRSystem databases), hsa-miR-23a (Ming et al. 2015a), hsa-miR-20a (Li et al. 2012a) and hsa-miR-221 (Hao et al. 2011). On the other hand, another study surprisingly found that 10 nM E_2 did not affect miRNA expression in T-47D cells after 24 h (Katchy et al. 2012). These authors reported 7 miRNA displaying tendencies of alteration ($p < 0.1$); however, qPCR experiments failed to confirm the effects.

1.6.2 Effect of progesterone on miRNA expression

T-47D cells have been exposed to the PR agonist medroxyprogesterone acetate at a concentration of 10 nM to evaluate its effect on the miRNA profile (Cochrane et al. 2012). It was found that after 6 h, a fold increase or decrease above 1.5 occurred in 28 miRNA, including hsa-miR-27b, hsa-miR-30c, hsa-miR-29b, hsa-miR-141, hsa-miR-374a, hsa-miR-30b, hsa-miR-29c, hsa-miR-130a, hsa-miR-30e, hsa-miR-101 and hsa-miR-20a. More recently, another study explored the role of P_4 in miRNA expression in the glioblastoma cell line U-251 using sequencing experiments (Velàzquez-Vàzquez et al. 2021). Here, P_4 regulated 16 miRNA including hsa-miR-452-5p, hsa-miR-197-5p and hsa-miR-485-3p. Interestingly, no common miRNA were altered between T-47D and U-251 cells in the two separate experiments.

1.6.3 Effect of prolactin on miRNA expression

Only a few studies have explored the effects of PRL on miRNA. One study found that 42 miRNA were altered by PRL treatment in T-47D cells (Wei et al. 2013). Another study investigating the effects of PRL on lipogenic miRNA in mammary epithelial cells found that four miRNA (miR-23a, miR-27b, miR-103 and miR-200a) were elevated in response to PRL, beginning at 2 µg/mL and most pronounced at 10 µg/mL (Lin et al. 2013). Further, some of those miRNA were predicted to be synergistically regulated, suggesting potential synergistic action of miRNA during PRL-associated lipogenesis. The concentration tested in both of these studies is above the physiological range of PRL. This is an issue because different genes can be activated by different dosage concentrations of the same hormone; thus, results of non-physiological exposure might not represent real tissue responses to hormones. Also, even if similar genes are activated by hormones, the magnitude of gene expression changes that ensue may be different at different concentrations.

1.6.4 Effect of oxytocin on miRNA expression

Information on OXT regulation of miRNA is minimal; no studies at the time of this project directly treated breast cells with this hormone and tested changes in the miRNA profile. However, in human myometrial tissue samples of women administered OXT to induce labor, expression of 12 miRNA, including hsa-miR-141-3p, were found to be altered compared with tissue samples analyzed prior to labour (Cook et al. 2015). OXT-induced expression change was confirmed for five miRNA by RT-qPCR (hsa-miR-146b-3p, hsa-miR-196b-3p, hsa-miR-223-3p, hsa-miR-873-

5p and hsa-miR-876-5p), and three miRNA alterations (miR-146a-5p, hsa-miR-196b-3p and hsa-miR-876-5p) were reproduced in primary myocytes treated with OXT (Cook et al. 2015).

1.7 Effect of Cx43 and miRNA on breast cancer

miRNA play important roles in mammary gland development, but importantly, this does not only through Cx43 regulation. Some groups of miRNA are elevated in the wallaby during lactation (Modepalli et al. 2014), and abundantly expressed in milk and important to its production (Li et al. 2016a). Others are involved in lipid metabolism in the mammary gland, such as miR-126, miR-145, miR-150 and miR-24 (Chu et al. 2017; Heinz et al. 2016; Wang et al. 2017; Wang et al. 2015), milk synthesis, such as miR-148a (Muroya et al. 2016) or other lactation processes, such as miR-29 and miR-486 (Bian et al. 2015; Li et al. 2015a). In addition, they are involved in the process of remodeling during involution (miR-424/503) (Rodriguez-Barrueco et al. 2017). Consequently, their dysregulation can also lead to the development of breast cancer. Over two million women are diagnosed with breast cancer worldwide, making it the most frequent cancer in women (Lukasiewicz et al. 2021). In addition, the survival rate five years after the diagnosis is 89.6% for non-metastasized and 75.4% for metastasized breast cancer (Lukasiewicz et al. 2021), showing that metastasis is an important factor and large-scale death still occurs from this disease. In addition, the disease disproportionately affects less developed countries (Lukasiewicz et al. 2021). For all these reasons, breast cancer remains a burden on individuals and takes a toll on healthcare systems.

Normal mammary gland development depends on GJIC, and so it was originally believed that connexins are tumour suppressors. However, subsequent research reports that its role is far more complex, being dependent on the sub-type and stage of breast cancer. For example, of the four intrinsic types of breast cancer, luminal A, luminal B, basal and Her2e, it has been demonstrated that Cx43 is over-expressed in unstratified and luminal tumours, but under-expressed in the Her2e subtype (Busby et al. 2018). Cx43 can act as a tumour suppressor, whereby decrease in its expression results in aberrant GJIC and increased cancer progression (Fentiman et al. 1977; Nicolas et al. 1978), and as an oncogene whose overexpression is associated with metastasis (el-Sabban et al. 1994; Elzarrad et al. 2008). In a broad sense, the role of miRNA in breast cancer progression is similar to Cx43. As miRNA regulate processes such as proliferation, differentiation, apoptosis and migration, it is unsurprising that dozens of cancer-linked miRNA showing aberrant upregulation or downregulation have been identified (Loh et al. 2019; Davey et al. 2021). miRNA are themselves transcribed from genes, so over or underactivation of gene regulation

mechanisms can lead to over or underexpression of their target proteins, affecting tumour growth. Most of the mechanistic roles of miRNA in breast cancer are unknown, though like Cx43 they can play the role of tumour suppressors or oncogenes.

1.8 Problematic, hypotheses and objectives

1.8.1 Problematic

Due to their association with breast cancer, it is of particular importance to understand the role of miRNA in mammary gland development and in Cx43 regulation to determine how they can contribute to its pathogenesis. Currently, it is unclear how miRNA contribute to mammary gland development, as they have been discovered relatively recently, and hundreds of different miRNA can each have multiple different targets and effects. Few studies exist showing the relationship between estradiol, progesterone, prolactin and oxytocin, on Cx43 nor miRNA. Further, even fewer of those existing studies are mammary gland studies, but analyze these hormones in other tissues. Thus, this project brings new and important insights on mammary gland development by addressing the aforementioned research gaps.

1.8.2 Hypotheses

The hypotheses at the base of this project are that miRNA vary in a stage-specific manner during mammary gland development and are associated with key hormones directing its morphogenesis. In addition, we hypothesize that miRNA are functional suppressors of Cx43 expression, and in this way can contribute to GJIC between cells within the luminal and myoepithelial layers.

1.8.3 Objectives

The global objectives of this project are to:

1) Characterize the variation in miRNA expression across different stages of mammary gland development *in vivo*.
2) Determine the effects *in vitro* of the key mammary gland hormones, estrogen, progesterone, prolactin and oxytocin on a) the expression of miRNA and b) the expression of the mRNA of Cx43.
3) Confirm the hormonal and miRNA-related regulation of Cx43 *in vitro*.

CHAPTER 2 : RESULTS PART 1

2.1 Article #1 introductory section

The objectives of this article are the following: 1) Analyze miRNA expression profiles across various stages of mammary gland development *in vivo*; 2) Determine the impact of hormones on the miRNA profile *in vitro;* 3) Assess whether variation in hormone levels between stages of development could be correlated with specific groups of miRNA; and 4) Predict potential gene targets of differentially expressed and hormonally regulated miRNA *in silico*.

The hypothesis of this article is the following: microRNA are differentially expressed across mouse development, and intricately linked with hormone expression at the different stages. Predicted targets of fluctuating and hormonally regulated miRNA are expected to be associated with transition between stages and hormone pathways, respectively.

To accomplish the objectives of this article, mammary glands were harvested from mice at W10, P18, Lac7 and Inv3 (N = 6) of development, and converted to powder under cryogenic conditions. Then, miRNA extraction and sequencing were performed on the samples. In addition, T-47D cells were cultured, then treated with estradiol, progesterone, prolactin and oxytocin. In the first round of exposures, proteins were extracted and markers analyzed by Western blot, to validate the effect of the hormones on downstream targets. In the second round of exposures, miRNA were extracted and sequenced (N = 3), to assess the effect of hormones on the miRNA profile in breast cells. For both *in vivo* and *in vitro* sequencing experiments, quality of all extracts was validated using a Nanodrop spectrophotometer and 2100 bioanalyzer.

Hormonal regulation of miRNA during mammary gland development

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Authors' contributions

CC: project plan, project methodology, project analyses, data interpretation, document revision

MJ: project design, project plan, project methodology, document revision

IP: project design, project plan, project methodology, data interpretation, project supervision, document redaction, document revision

2.2 Abstract

The mammary gland is a unique organ as most of its development occurs after birth through stages of proliferation, differentiation and apoptosis that are tightly regulated by circulating hormones and growth factors. Throughout development, hormonal cues induce the regulation of different pathways, ultimately leading to differential transcription and expression of genes involved in this process, but also in the activation or inhibition of post-transcriptional mechanisms of regulation. However, the role of microRNAs (miRNAs) in the different phases of mammary gland remodeling is still poorly understood. The objectives of this study were to analyze the expression of miRNA in key stages of mammary gland development in mice and to determine if it could be associated with hormonal variation between stages. To do so, miRNAs were isolated from mouse mammary glands at stages of adulthood, pregnancy, lactation and involution, and sequenced. Results showed that 490, 473, 419, and 460 miRNAs are detected in adult, pregnant, lactating and involuting mice, respectively, most of them being common to all four groups, and 58 unique to one stage. Most genes could be divided into 6 clusters of expression, including 2 encompassing the highest number of miRNA (cluster 1 and 3) and showing opposite profiles of expression, reaching a peak at adulthood and valley at lactation, or showing the lowest expression at adulthood and peaking at lactation. GO and KEGG analyses suggest that the miRNAs differentially expressed between stages influence the expression of targets associated with mammary gland homeostasis and hormone regulation. To further understand the links between miRNA expression and hormones involved in mammary gland development, miRNAs were then sequenced in breast cells exposed to estradiol, progesterone, prolactin and oxytocin. 38, 4, 24 and 66 miRNAs were associated with estradiol, progesterone, prolactin, and oxytocin exposure, respectively. Finally, when looking at miRNAs modulated by the hormones, differentially expressed during mammary gland development, and having a pattern of expression that could be correlated with the relative levels of hormones known to be found *in vivo,* 16 miRNAs were identified as likely regulated by circulating hormones. Overall, our study brings a better understanding of the regulation of miRNAs throughout mammary gland development and suggests that there is a relationship with their expression and main hormones involved in mammary gland development. Future studies will examine this role more in detail.

2.3 Introduction

The mammary gland is a unique organ as most of its development occurs after birth through stages of proliferation, differentiation and apoptosis that are tightly regulated by circulating hormones and growth factors (Figure 1). Studies mainly using transplants and genetically engineered mice have demonstrated that some of these hormones play a crucial role in mammary gland development and function in a stage-specific manner, while others play accessory or less understood roles [1]. It is composed of two main compartments: the epithelium, a ramified tree-like structure that produces milk during lactation, and the stroma (also called fat pad), mainly composed of adipocytes, fibroblasts, immune cells and extracellular matrix [2, 3]. At birth, the rudimentary epithelial tree is composed of only a few branches that grow at the same pace as the body until puberty (Figure 1). Then, a surge of hormones, particularly estrogens, induces the proliferation of epithelial cells at the tips of ducts, in structures named terminal-end buds (TEBs) [4]. Between puberty and adulthood, the epithelium continues to elongate and ramify, mainly under the combined action of estrogens and progesterone, until it reaches the edge of the fat pad [1, 5]. The mammary gland then undergoes cycles of proliferation and apoptosis, in accordance with the hormonal variation induced by each menstrual cycle. Another intense phase of remodeling occurs during pregnancy with the rising levels of prolactin and progesterone [1, 5-9], whereby the number of secondary and tertiary branches increases drastically, providing a ductal arbor for the formation of alveoli, the units that secrete milk. After parturition, the withdrawal of progesterone triggers secretory activation. Prolactin stimulates the production of milk, while oxytocin stimulates milk ejection [1, 5]. At weaning, milk accumulation and change in hormonal stimulation, notably a decrease in prolactin and an increase in estrogens, induces involution of the mammary gland epithelium [10], whereby the mammary gland gradually returns to a structure similar to its pre-pregnancy stage. Of note, during all these phases, the stroma is also remodeled and actively contributes to the development of the epithelium by providing chemical, physical, and nutritional support, although mechanisms involved in this process are less known. Throughout these phases of remodeling, hormonal cues induce the regulation of different pathways, ultimately leading to differential transcription and expression of genes involved in this process, but also in the activation or inhibition of post-transcriptional mechanisms of regulation.

MicroRNAs (miRNAs) are 19 to 25 nucleotides long non-coding RNA molecules that post-transcriptionally regulate the expression of messenger RNA (mRNA), typically by binding to consensus sequences on their 3'-UTR [11]. Over the last decades, thousands of miRNAs have been identified and classified into families, based on their seed sequences, i.e. primary

determinant of recognition for miRNAs [11]. Increasing number of articles are reporting the crucial role that miRNAs play in regulating the expression of genes within tissues, which regulates important tissue functions such as development, differentiation, proliferation, neoplastic transformation, and apoptosis [11]. As a result, it is generally assumed that high levels of a specific miRNA within a cell or a tissue likely reflect a regulatory role, and that increased expression upon change in conditions is likely to have a biological impact on that tissue [12]. Thus, evaluating the expression of miRNA during different stages of mammary gland development can bring important insights on their stage-specific role, and also on the impact of hormones on their regulation or on their role in breast cancer.

Compared to increasing literature reporting the differential expression of specific miRNAs in breast cancer tissues compared to normal tissues, only a few studies have evaluated the role of miRNA in mammary gland development. Using a bead-based flow-cytometric microarray platform, 102 miRNAs were detected in mouse mammary gland tissues from 18 different time points [13]. The expression of miRNA was found to follow 7 clusters of expression, and the mean miRNA expression was lower at lactation [13]. Although this study provided an initial screen of miRNA expression in the mammary gland, it did not allow for a global-scale analysis of the miRNAome. Similarly, the expression of miRNA was analyzed in breast tissues from human, cow, goat and other animals but mainly during lactation [14-23]. Other studies have focused on specific miRNA or a small number of miRNAs [24-26]. Interestingly, in a recent literature review, 32 miRNA were identified as implicated in both mammary gland development and breast cancer development across species, and were associated with specific stages of development or tumorigenic processes [27].

Given the crucial role of miRNA in the development and function of tissues, a deeper analysis of miRNA expression during mammary gland development is required to further understand their role. In addition, although the development of the mammary gland is tightly orchestrated by hormones, little is known regarding the relationship between miRNA and hormones during mammary gland development. The objectives of this study were to analyze transcriptome-wide changes in the expression of miRNAs in key stages of mammary gland development in mice and to determine whether regulation of specific miRNA subsets could be associated with hormonal variation between stages.

2.4 Materials and methods

2.4.1 Animals

Female C57BL/6 mice were purchased from Charles River Canada (St. Constant, Quebec, Canada). Mice were maintained under a constant photoperiod of 12h light:12h dark and received food and water ad libitum. All animal protocols used in this study were approved by the University Animal Care Committee (INRS-Armand-Frappier Santé Biotechnologie, Laval, Canada). Female mice were sacrificed using CO_2 followed by cardiac puncture, and the mammary glands were collected at the following time points: adult (W10), pregnancy day 18 (P18), lactation day 7 (Lac7), and involution day 3 (Inv3). These stages were chosen based on a previous study that showed that miRNA expression is similar from 6 weeks of age to early pregnancy, as for lactation and early stages of involution (between 12-48h after weaning) [13]. We thus used 4 distinct stages to 1) optimize the distinction between miRNAs expressed at each stage, and 2) to get different hormonal and stage profiles. For each developmental stage, 6 mice were sampled (N=6). The mammary gland pairs 4 and 5 (abdominal and inguinal) were flash-frozen in liquid nitrogen immediately after dissection.

2.4.2 Cell culture and treatments

T47D cells, a luminal breast cancer cell line originating from a 54-year-old female patient, were obtained from American Type Culture Collection (ATCC). Cells were maintained in Roswell Park Memorial Institute (RPMI-1640) media without phenol red (Thermo Fisher, USA), supplemented with heat inactivated fetal bovine serum (FBS) (Wisent Bio Products, CAN) at 10% final concentration, and incubated at 37°C and 5% CO_2. Cells were plated at normal seeding density and passaged before 90% confluency to maintain log-phase growth. Doubling time was calculated and the cells were not kept for more than 10 passages. At 60 to 70% confluency and 16 hours prior to hormonal exposure, media was replaced with media containing hormone-stripped FBS (Wisent Bio Products, CAN) to eliminate effects of background hormone signaling. Then, cells were exposed to estrogen (E8875, Sigma-Aldrich, USA), progesterone (P0130, Sigma-Aldrich, USA), prolactin (L4021, Sigma-Aldrich, USA) and oxytocin (O6379, Sigma-Aldrich, USA) or the appropriate vehicle. Based on literature, treatments were as follows: 8.7 nM (200 ng/mL) and 8 hours for prolactin, 10 nM and 24 hours for estrogen, 10 nM and 6 hours for progesterone, and 100 nM and 24 hours for oxytocin [28-31]. Vehicles were 0.1% ethanol for estrogen and progesterone, 4 mM hydrochloric acid for prolactin and nuclease-free water for oxytocin.

2.4.3 *In vitro* miRNA isolation and sequencing

Upon termination of exposure time, media was removed, cells were washed with Dulbecco's phosphate buffered saline (Thermo Fisher, USA) and detached by incubating at 37°C with trypsin-EDTA 0.25% (Thermo Fisher, USA) for 7 to 10 minutes. Then, cells were pelleted by centrifuging for 5 minutes at 125 g, and the supernatant was discarded. The *mir*Vana™ miRNA isolation kit (Thermo Fisher Scientific, Mississauga, ON, Canada) was used according to instructions to isolate small RNA samples from cells. miRNAs were quantified using a Nanodrop 1000 (Thermo Fisher, USA), while purity and integrity were assessed using the Agilent Small RNA kit and an Agilent 2100 bioanalyzer (Agilent, USA). Data were analyzed using the Bioanalyzer 2100 Expert (version B.02.11.S1824 SR1) software; digital gel images and electropherograms of samples were validated prior to sequencing. Extracts were delivered to the Institute for Research in Cancer and Immunology (IRIC) for miRNA-seq analysis (Illumina platform). FASTQC v0.11.8 was used for read quality assessment, while Cutadept was used for reads trimming. miRNA amount was calculated using MIRDEEP and COMPSRA software, and DESeq2 software was applied to calculate differential expression within each separate hormone experiment. Where applicable, the removeBatchEffect software from limma was used to correct miRNA quantity.

2.4.4 *In vivo* miRNA isolation and sequencing

Frozen tissues were ground in liquid nitrogen. miRNAs were extracted from the resulting powder using the *mir*Vana™ miRNA isolation kit following the manufacturer's instructions (Thermo Fisher Scientific, Mississauga, ON, Canada). The miRNA concentration was quantified with a Nanodrop 1000 (Thermo Fisher Scientific), while purity and integrity were assessed using the Agilent Small RNA kit (Agilent, Wilmington, DE, USA) and an Agilent bioanalyzer 2100. Data were analyzed using the Bioanalyzer 2100 Expert (version B.02.11.S1824 SR1) software; digital gel images and electropherograms of samples were validated prior to sequencing. miRNA libraries were prepared using a QIAseq miRNA stranded kit (Quiagen, Toronto, ON, Canada) and sequenced using an Illumina NextSeq 500 apparatus. The mapping against reference genome was done using QuickMirSEq and miRbase/22 [32, 33]. Analyses of differently expressed miRNAs and of clustering were performed using DESeq2/1.26.0, DEGReport and bigPint applications [34, 35]

2.5 Results

2.5.1 miRNA expression follows 6 clusters of expression during mammary gland development

miRNAs were extracted and sequenced from mammary glands of adult, pregnant, lactating and involuting mice (Figure 2). Results showed that 490, 473, 419 and 460 miRNAs are detected in adult, pregnant, lactating and involuting mice (Supplementary tables 1-4) respectively, including 374, 66 and 45 expressed at four (all), three and two stages, respectively, and 58 unique to one stage (Figure 2 and Supplementary table 5). When analyzing the expression of individual miRNAs between the stages, most genes could be divided in 6 clusters of expression (Figure 3 and Supplementary table 6). Interestingly, the two clusters containing the highest number of miRNA (cluster 1 and 3) showed opposite profiles of expression. Cluster 1 showed an increase in miRNAs expression between adult and lactation, followed by a decrease at involution, while cluster 3 showed a decrease in miRNAs expression between adult and lactation, followed by an increase at involution (Figure 3). Cluster 4 also showed a decreased in miRNA expression specifically during pregnancy and lactation (Figure 3).

2.5.2 miRNAs vary in expression between different stages of mammary gland development

We next asked more precisely how miRNA expression fluctuates during mammary gland development by comparing consecutive stages of development. To do so, we identified miRNA differentially expressed in mammary glands from adult versus pregnant mice (Figure 4A and Supplementary table 7), between pregnant and lactating mice (Figure 4B and Supplementary table 8) and between lactating and involuting mice (Figure 4C and Supplementary table 9). 144, 165 and 167 miRNAs were differentially expressed between adult and pregnant glands, between pregnant and lactating glands, and between lactating and involuting glands, respectively (Figure 4 and Supplementary tables 7-9). For the first two comparisons, about 55% of those miRNAs were up-regulated, and 23 out of the 30 miRNAs that showed the highest fold change were also up-regulated (Figure 4A, B). However, an opposite trend was observed between lactating and involuting glands, as 57% of total differentially expressed miRNAs, and 24 out of the 30 miRNAs with the highest fold change, were down-regulated (Figure 4C and Supplementary table 9).

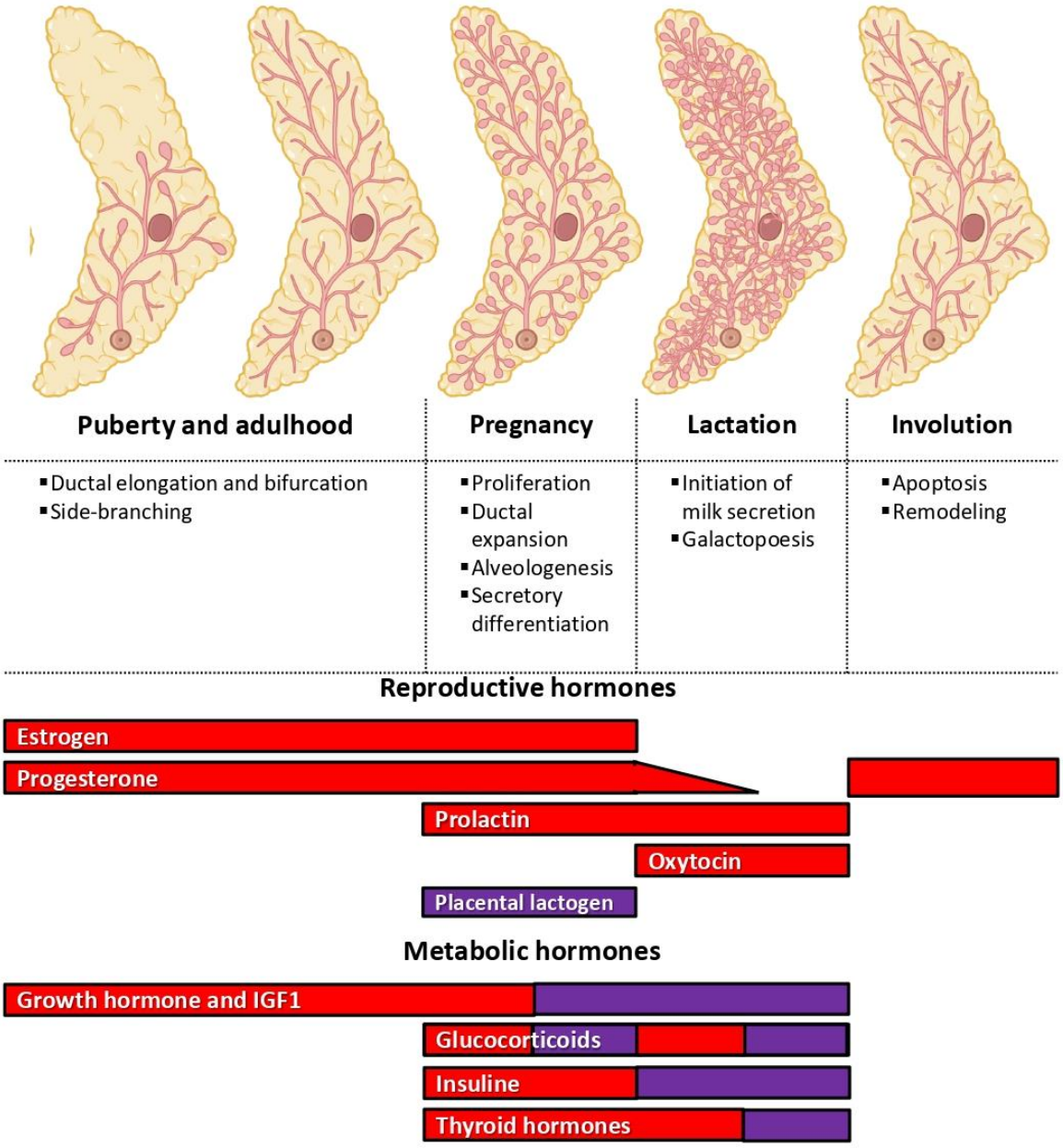
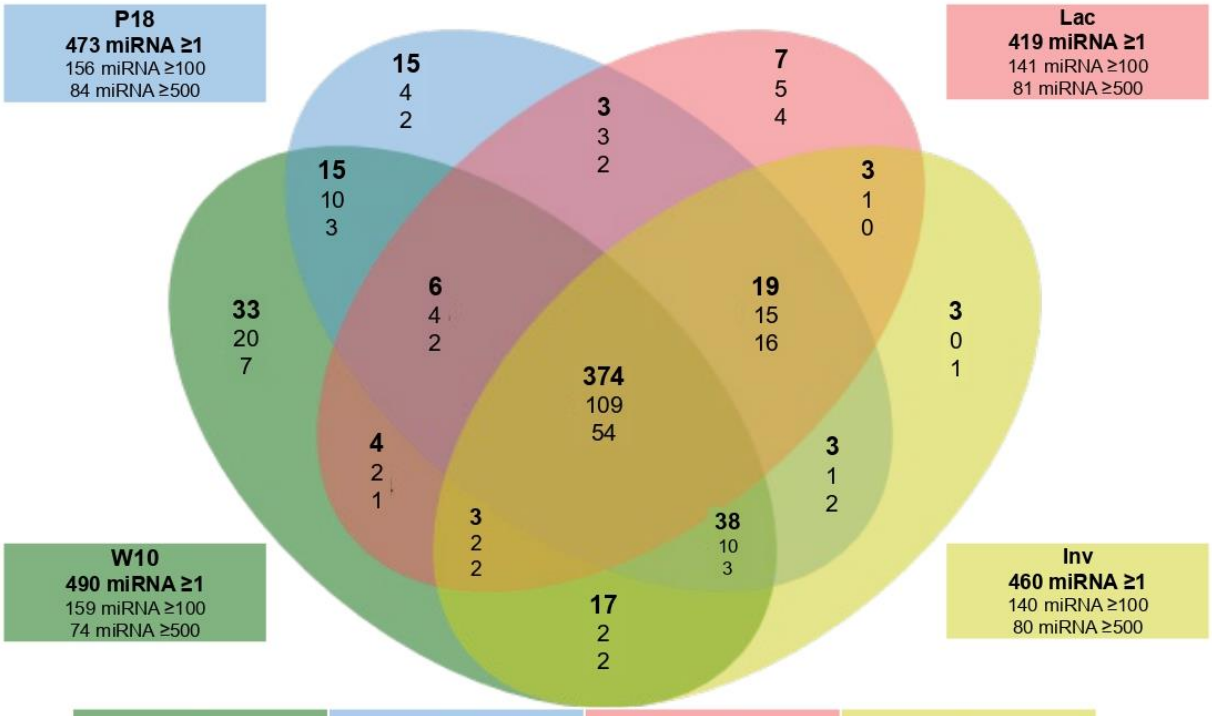


Figure 1: Main hormones involved in mammary gland development. The development of the mammary gland epithelium occurs mainly after birth. At puberty, a surge of hormones induces ductal elongation and bifurcation until the epithelium reaches the edge of the fat pad (i.e, stroma). During adulthood, the epithelium undergoes cycles of development and regression, following the menstrual cycles. A second wave of major remodeling will happen during pregnancy to form the alveoli that will produce milk during lactation. At weaning, the accumulation of milk will induce the involution of the epithelium to its pre-pregnancy stage. Reproductive and metabolic hormones have stage-specific roles during those processes, some having well-documented essential roles (red), and some having either complementary or less-documented roles (purple).



W10 33 miRNA	P18 15 miRNA	Lac7 7 miRNA	Inv 3 miRNA
mmu-miR-1a-1-5p	mmu-miR-23b-5p	mmu-miR-7116-5p	mmu-miR-3085-5p
mmu-miR-384-3p	mmu-miR-7083-5p	mmu-miR-222-5p	mmu-miR-1960
mmu-miR-669a-3-3p	mmu-miR-323-3p	mmu-miR-1947-5p	mmu-miR-5129-3p
mmu-miR-466-5p@	mmu-miR-380-3p	mmu-miR-421-5p	
mmu-miR-218-2-3p	mmu-miR-505-3p	mmu-miR-709	
mmu-miR-466m-5p@	mmu-miR-24-1-5p	mmu-miR-466j	
mmu-miR-218-2-3p	mmu-miR-7059-5p	mmu-miR-7063-5p	
mmu-miR-653-5p	mmu-miR-412-5p		
mmu-miR-5107-5p	mmu-miR-19-b-1-5p		
mmu-miR-6541	mmu-miR-19a-5p		
mmu-miR-1943-5p	mmu-miR-802-5p		
mmu-miR-466m-3p	mmu-miR-6951-5p		
mmu-miR-344-3p	mmu-miR-706		
mmu-miR-669b-5p	mmu-miR-32-3p		
mmu-miR-329-3p	mmu-miR-3081-3p		
mmu-miR-449a-5p			
mmu-miR-382-3p			
mmu-miR-467e-3p			
mmu-miR-467c-3p			
mmu-miR-218-1-3p			
mmu-miR-3060-3p			
mmu-miR-377-3p			
mmu-miR-466c-5p			
mmu-miR-541-3p			
mmu-miR-1948-3p			
mmu-miR-1964-3p			
mmu-miR-3064-3p			
mmu-miR-193a-3p			
mmu-miR-873a-5p			
mmu-miR-669b-3p			
mmu-miR-466a-5p@			
mmu-miR-188-3p			
mmu-miR-666-5p			
mmu-miR-466n-3p			
mmu-miR-129-2-3p			

Figure 2: miRNA expressed at 10 weeks of age (W10), day 18 of gestation (P18), day 7 of lactation (Lac7) and day 3 of involution (Inv3) in the mammary gland of mice. Small RNA were extracted from mammary glands using the mirVana™ miRNA Isolation kit (ThermoFisher) and sequenced (Illumina NovaSeq6000). 6 animals were used per group (N=6). Between 74 and 490 miRNAs were identified per group, depending of the threshold of average reads per million per group (>1, upper number; > 100 middle number; > 500 lower number, within the text boxes). The table lists the miRNA that are unique for each stage.

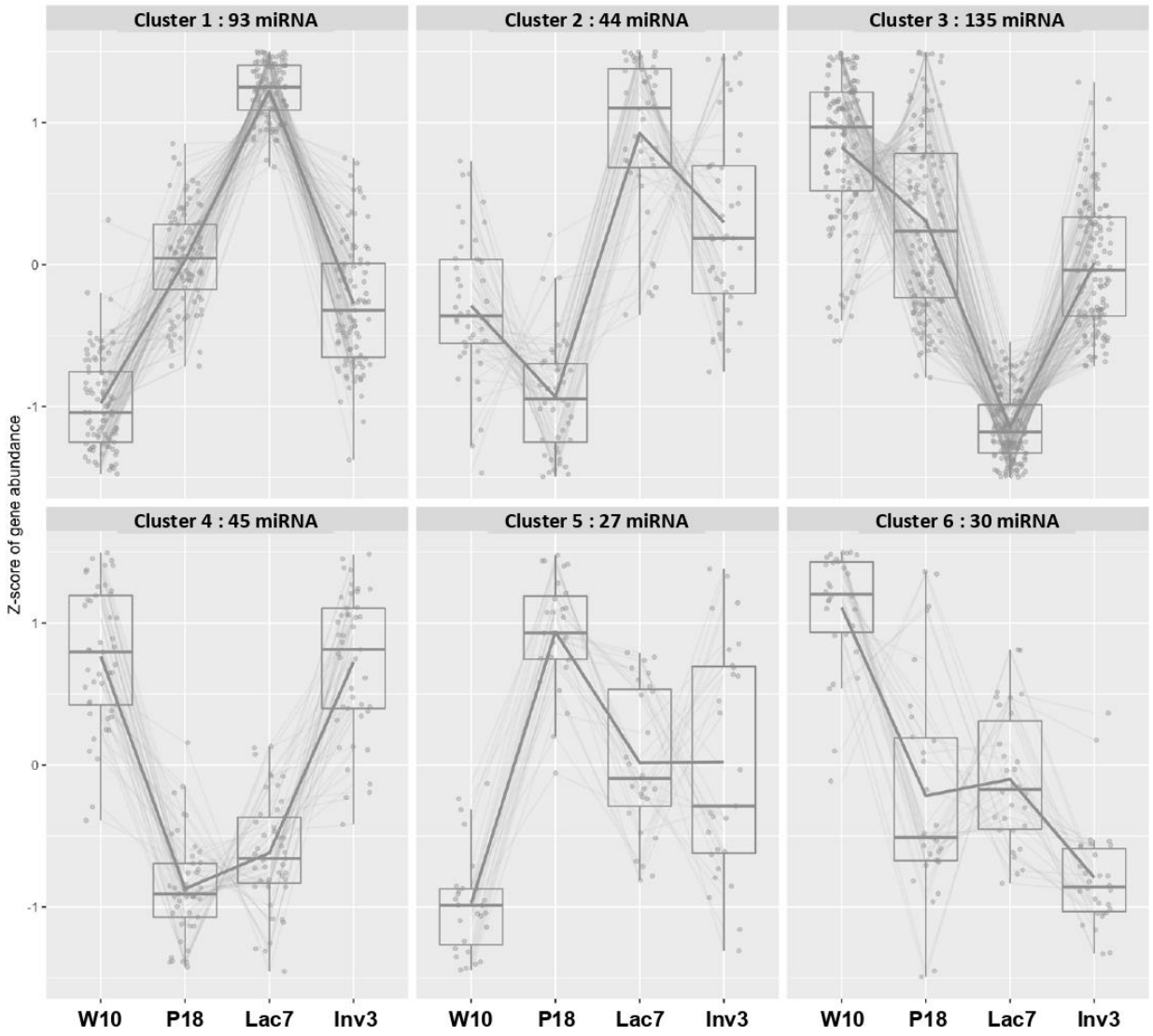


Figure 3: miRNA expressed in the mammary gland can be clustered in 6 patterns of expression. Using bigPint library (Rutter, 2020; Stephens, 2017) 6 clusters of expression were identified, comprising 93, 44, 135, 45, 27 and 30 miRNA, respectively.

2.5.3 Differentially expressed miRNAs are predicted to target various biological processes and pathways

To better understand how the change in miRNAs expression could be linked with biological processes, genes predicted to be targeted by those miRNAs were identified using miRDBv6.0 and used in a Gene Ontology (GO) enrichment (Supplementary figure 1 and tables 10-12) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (Figure 5). A

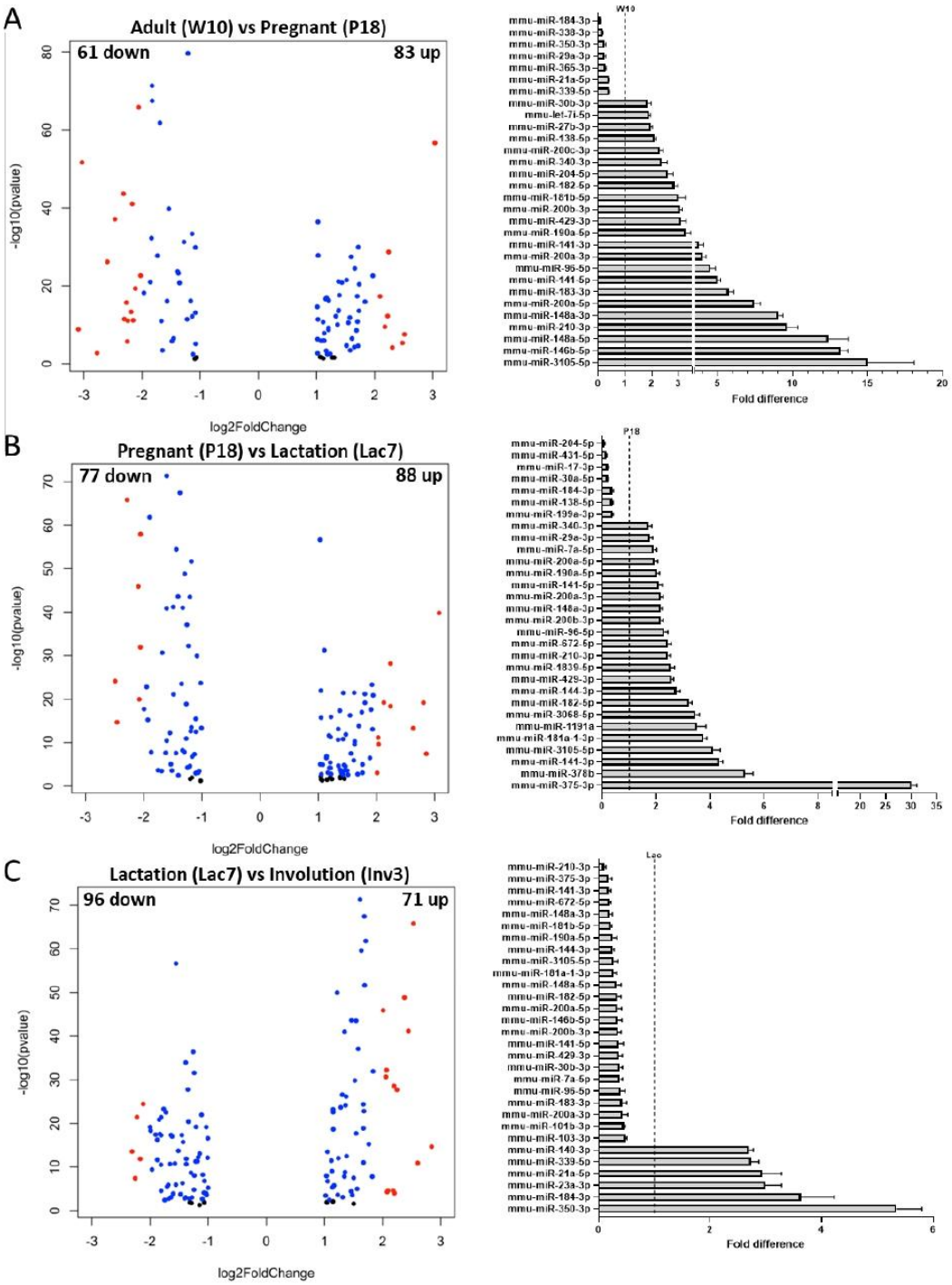


Figure 4: miRNA differentially expressed between two consecutive stages of mammary gland development. miRNA differentially expressed between (A) adult and pregnant, (B) pregnant and lactating, and (C) lactating and involuting mice were identified using the *DESeq2/1.26.0* application (Log 2 fold change cutoff = 1; FDR cutoff: 1). The Volcano plots (left) show up- and down-regulated miRNA for each comparison; the 30 miRNA that showed the biggest fold change are showed on the right. On volcano plots, red dots showed miRNA with Log 2 fold change (- or +) ≥ 2, while blue dots represent miRNA with Log 2 fold change (- or +) between 1 and 2. Black dots represent miRNA with p values between 0.05 and 0.001.

similar number of predicted genes were identified for each comparison, with 4999, 4414 and 5046 genes predicted to be targeted by the miRNAs between adult and pregnant mice, pregnant and lactating mice, and lactating and involuting mice, respectively (Supplementary figure 1 and tables 10-12).

Using KEGG pathway enrichment analysis, we identified the 10 most enriched pathways involving the predicted targets. 6 were common to the three comparisons: “Breast cancer”, “FoxO signaling pathway”, “Axon guidance”, “Pathways in cancer”, “MAPK signaling pathway”, and “PI3K-Akt signaling pathway” (Figure 5). “Focal adhesion” was found when comparing adult and pregnant mice, and pregnant and lactating mice, while “Prostate cancer” was common to the comparisons between adult and pregnant mice, and pregnant and lactating mice (Figure 5). “Endocrine resistance”, “Choline metabolism in cancer“ and “Relaxin signaling pathway” were only found between adult and pregnant mice; “Regulation of actin cytoskeleton” and “Chronic myeloid leukemia” were only found between pregnant and lactating mice; “Signaling pathways regulating pluripotency of stem cells”, “Hedgehog pathway” and “Proteoglycans in cancer” were found only when comparing lactating and involuting mice (Figure 5).

2.5.4 Various genes predicted to be targeted by expressed miRNA can be associated with hormonal regulation

From the KEGG pathway enrichment analysis, a few pathways could be linked with mammary gland homeostasis and/or hormone regulation, such as “Breast cancer”, and “Endocrine resistance” (Figure 5). Other pathways, such as the FoxO, PI3K-Akt and MAPK pathways are known to crosstalk with the hormonal pathways [36-40]. Knowing the importance of hormonal signaling in mammary gland development (Figure 1), we then asked whether changes in miRNAs expression can be correlated with activation or inhibition of hormonal signaling pathways. To do so, we first search the lists of predicted genes obtained by miRDBv6.0 for each comparison groups using the terms “estrogen, progesterone, prolactin, placental, oxytocin, growth hormone, insulin-like, glucocorticoid, insulin and thyroid” in reference with the main hormones shown to be involved in mammary gland development (Figure 1). 36, 32 and 41 genes predicted to be targeted by miRNA were found when analyzing results from the 3 comparisons groups, including 22 that were common to all, 15 shared by two groups of comparison and 13 that were unique to a specific group of comparison (Figure 6). For each comparison, most genes found were related with estrogen, insulin-like or insulin, and thyroid (Figure 6, table).

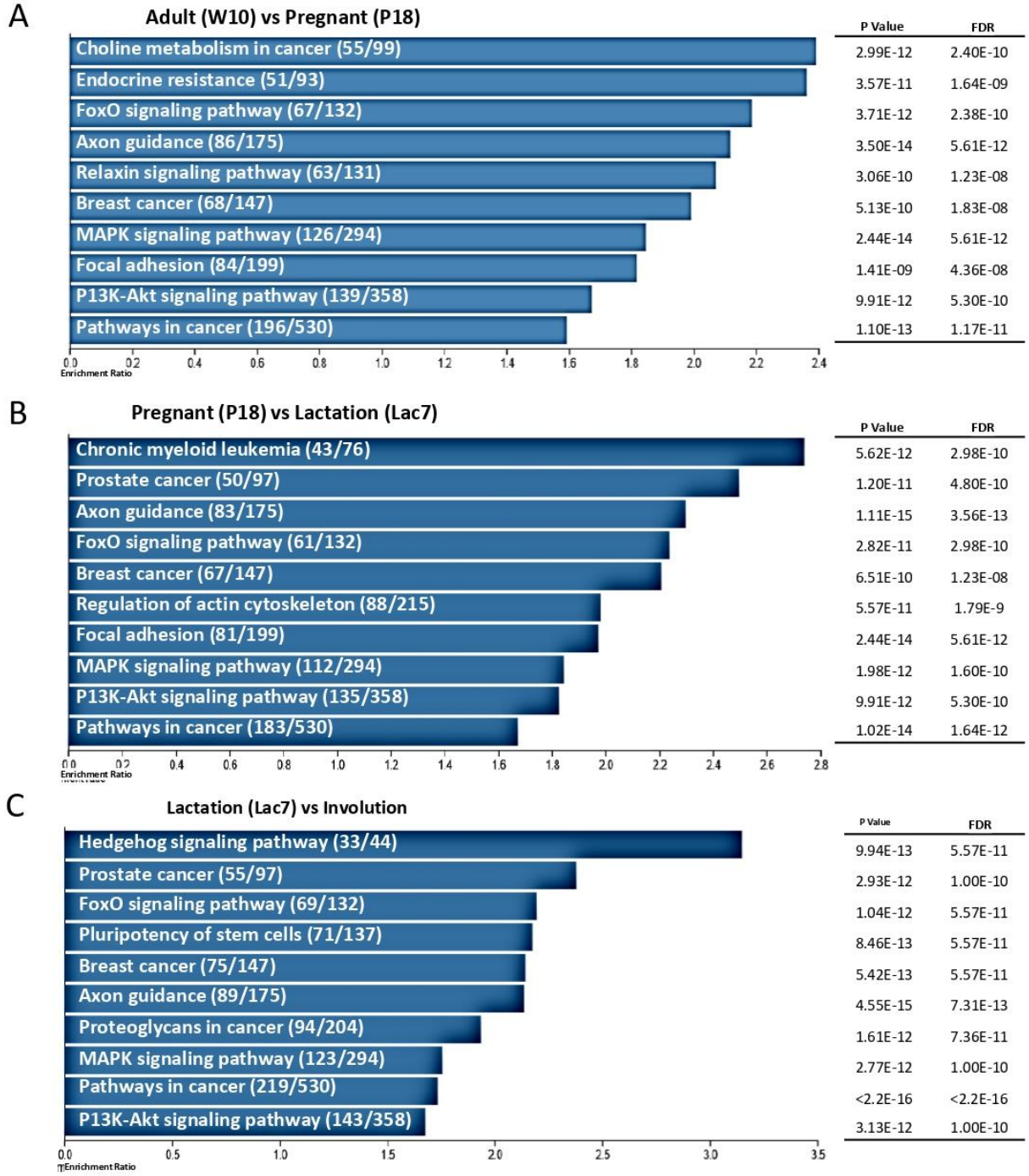
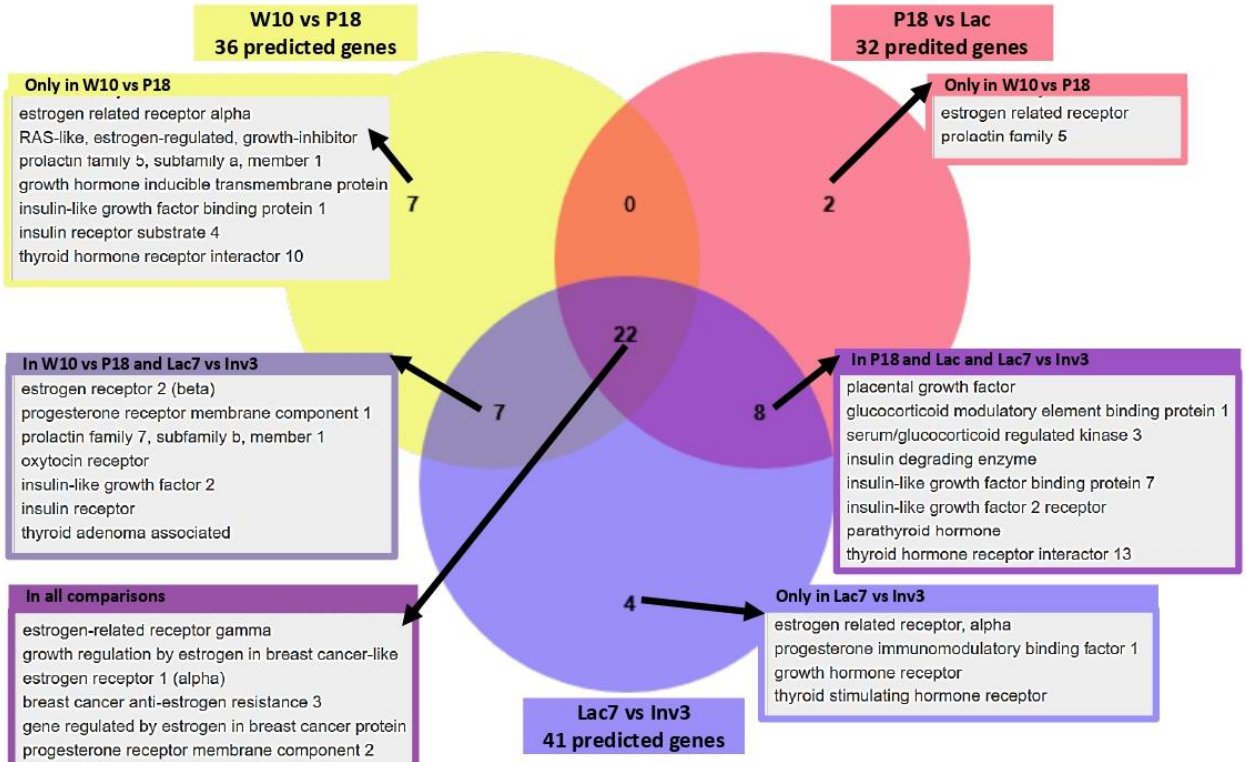


Figure 5: Predicted biological processes and pathways targeted by the differentially expressed miRNAs. The list of predicted mRNA target was generated for each set of differentially expressed miRNA ((A) adult vs pregnant; (B) pregnant vs lactating; (C) lactating vs involuting mice) using miRDB/v6.0. These lists of predicted targets were analyzed using a KEGG pathway enrichment analysis. The top 10 pathways predicted to be modified are showed for each comparison. Numbers in brackets = number of overlap/gene set size.



Hormone	W10 vs P18	P18 vs Lac7	Lac7 vs Inv3
Estrogen	8	6	7
Progesterone	2	1	3
Prolactin	3	2	2
Placental	0	1	1
Oxytocin	1	0	1
Growth hormone	1	0	1
Insulin-like	8	8	9
Glucoroticoid	2	4	4
Insulin	5	4	5
Thyroid	6	6	8

Figure 6: Genes associated with hormonal regulation predicted to be modulated by the differentially expressed miRNA for each comparison. The list of predicted mRNA target was generated for each set of differentially expressed miRNA ((A) adult vs pregnant; (B) pregnant vs lactating; (C) lactating vs involuting mice) using miRDB/v6.0. and search for the terms: estrogen, progesterone, prolactin, placental, oxytocin, growth hormone, insulin-like, glucocorticoid, insulin and thyroid in reference to the main hormones known to be implicated in mammary gland development (Figure 1). The Venn diagram shows results for each comparison and common to 2-3 comparisons. The table shows the number of terms associated with each hormone for each comparison.

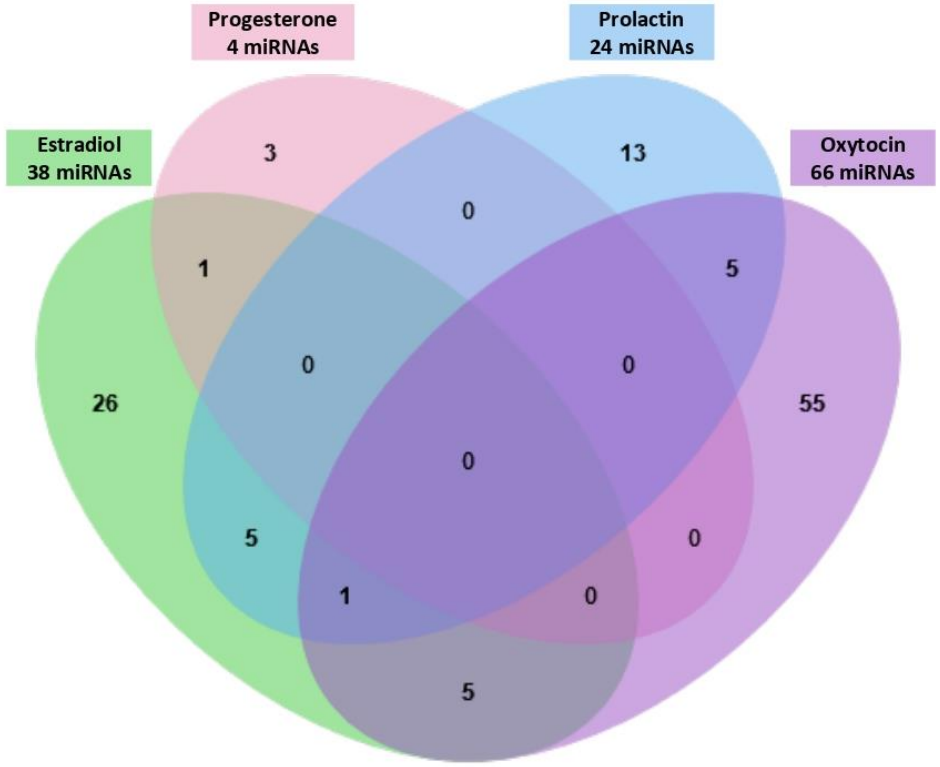
2.5.5 Distinct miRNAs are regulated by hormones *in vitro*

To further understand the links between hormones and miRNAs, we next exposed human breast cells to the 4 reproductive hormones that are well known to have a crucial role in mammary gland development and function, namely estradiol (estrogen), progesterone, prolactin, and oxytocin (Figure 1). miRNAs were extracted and sequenced to evaluate the effects of hormonal treatment on miRNA expression. 38, 4, 24 and 66 miRNAs were associated with estradiol, progesterone, prolactin, and oxytocin, respectively (Figure 7 and supplementary table 13). Interestingly, no miRNA was common to all hormones, and most of them were unique to a specific treatment.

2.5.6 Distinct miRNAs expressed in mice are hormonally regulated in breast cells

We next wanted to determine whether some miRNAs found *in vivo* could be linked with the 4 reproductive hormones tested *in vitro.* We thus compared the differentially expressed miRNAs upon each treatment (Figure 7) with the list of miRNAs found at each stage of development (Figure 2). Since it has been suggested that miRNA representing less than 100 reads per million are unlikely to be functionally relevant [41], only miRNA with reads above 500 were used for each development stage. For estradiol, out of the 38 miRNAs that were statistically differentially expressed by the treatment *in vitro*, 5 were expressed at the four developmental stages (Figure 8) while miR-92a-3p was expressed only in adult and pregnant mice, miR-342-3p was expressed in pregnant and involuting mice, miR-181b-5p was expressed in pregnant and lactating mice, and miR-205-5p in involuting mice. For progesterone, only 4 miRNAs were found to be modulated by progesterone *in vitro,* and none were found to be expressed in the mammary gland at the stages studied (Figure 9). For prolactin, 6 out of 24 miRNAs were expressed at the four developmental stages, and 2 were found in all stages, except adult mice (Figure 10). Surprisingly, miRNAs linked with oxytocin, which is thought to be crucial only at lactation (Figure 1), showed the most variations. Out of the 66 miRNAs differentially expressed *in vitro*, 5 were common to all stages of development and 4 were found in all stages, except adult (Figure 11). miR-92a-3p was expressed only in adult and pregnant mice, and miR-27a-3p and miR-29b-3p were expressed in adult and involuting mice only (Figure 11).

Using the miRNAs identified from the Venn diagrams (Figures 8-11), we then evaluated whether their patterns of expression could be correlated with the relative levels of hormones known to be found *in vivo* (Figures 12-15). Based on these analyses, our results suggest that estradiol and prolactin are likely to influence the expression 4 miRNAs each during mammary gland



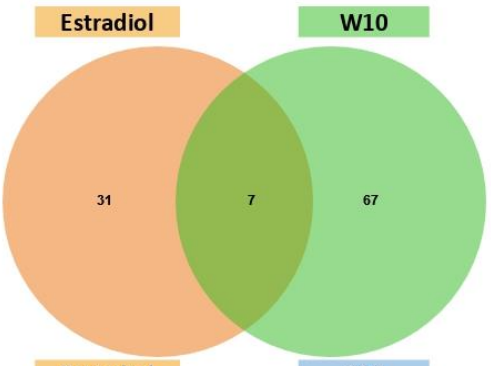
	Estradiol	Progesterone	Prolactin	Oxytocin
Up	20 (53%)	3 (75%)	15 (63%)	29 (44%)
down	18 (47%)	1 (25%)	9 (37%)	37 (56%)

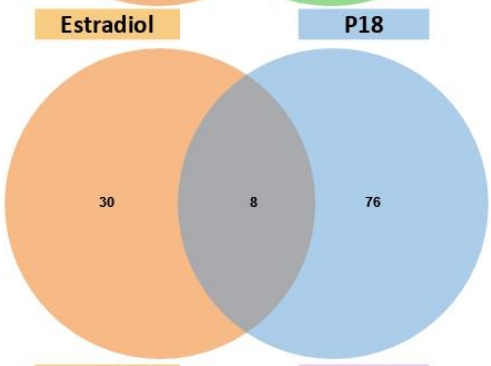
Figure 7: miRNA modulated by estradiol, progesterone, prolactin and oxytocin in human breast T47D cells. T47D cells were exposed to estradiol, progesterone, prolactin and oxytocin. Small RNA were extracted from cells using the mirVana™ miRNA Isolation kit (ThermoFisher) and sequenced (Illumina NovaSeq6000). 38 (estradiol), 4 (progesterone), 24 (prolactin) and 66 (oxytocin) miRNA were differentially expressed in treated cells compared to vehicle treated cells. The Venn diagram shows that most miRNA are unique to one treatment and none are shared by all treatments.

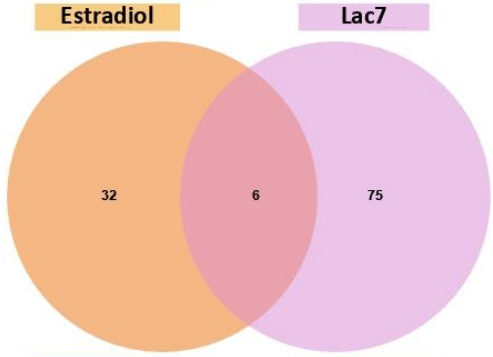
development (miR-181b-5p, miR-25-3p, miR-378a-3p, miR-92a-3p; miR-16-5p, miR-125b-5, let-7d-5p, miR-148a-3p), while oxytocin seems to regulate 8 miRNA (miR-125a-5p, miR-92a-3p, miR-200a-3p, let-7f-5p, miR-141-3p, miR-141-5p, miR-148a-3p, miR-32-5p) during lactation (Figure 15).

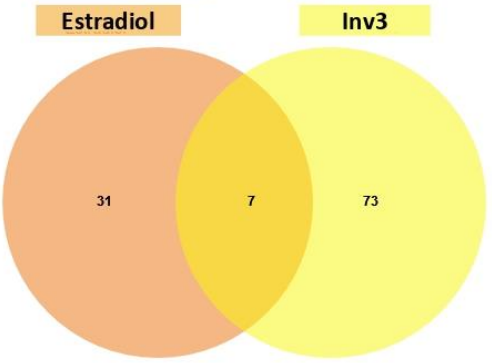
2.6 Discussion

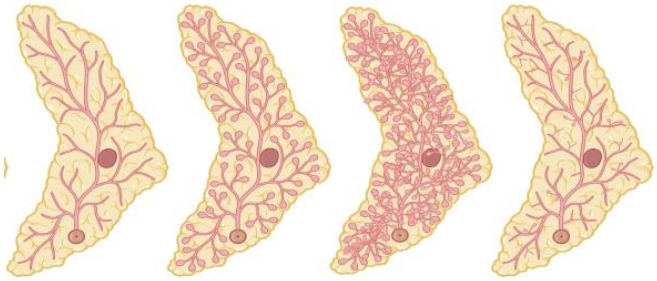
In the current study, we showed that although many miRNAs are expressed at the 4 key stages of mammary gland development studied and a few are uniquely expressed at one stage, their

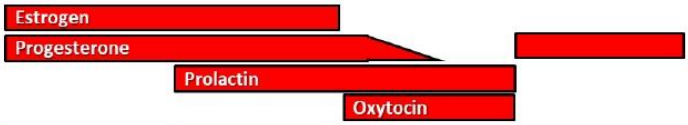












W10	P18	Lac7	Inv3
miR-125b-5p	miR-125b-5p	miR-125b-5p	miR-125b-5p
miR-23b-3p	miR-23b-3p	miR-23b-3p	miR-23b-3p
miR-30c-5p	miR-30c-5p	miR-30c-5p	miR-30c-5p
miR-25-3p	miR-25-3p	miR-25-3p	miR-25-3p
miR-378a-3p	miR-378a-3p	miR-378a-3p	miR-378a-3p
miR-92a-3p	miR-92a-3p		
miR-99a-5p			
	miR-342-3p		miR-342-3p
	miR-181b-5p	miR-181b-5p	
			miR-205-5p

Figure 8: Comparison between miRNA expressed at each stage of development and miRNA modulated by estradiol. The Venn diagram shows that between 6-8 miRNAs that are modulated by estradiol are expressed at the four stages of development studied (≥.500 reads). The table shows the list of miRNA that were up-regulated (green) and down-regulated (red) by estradiol at each stage of development.

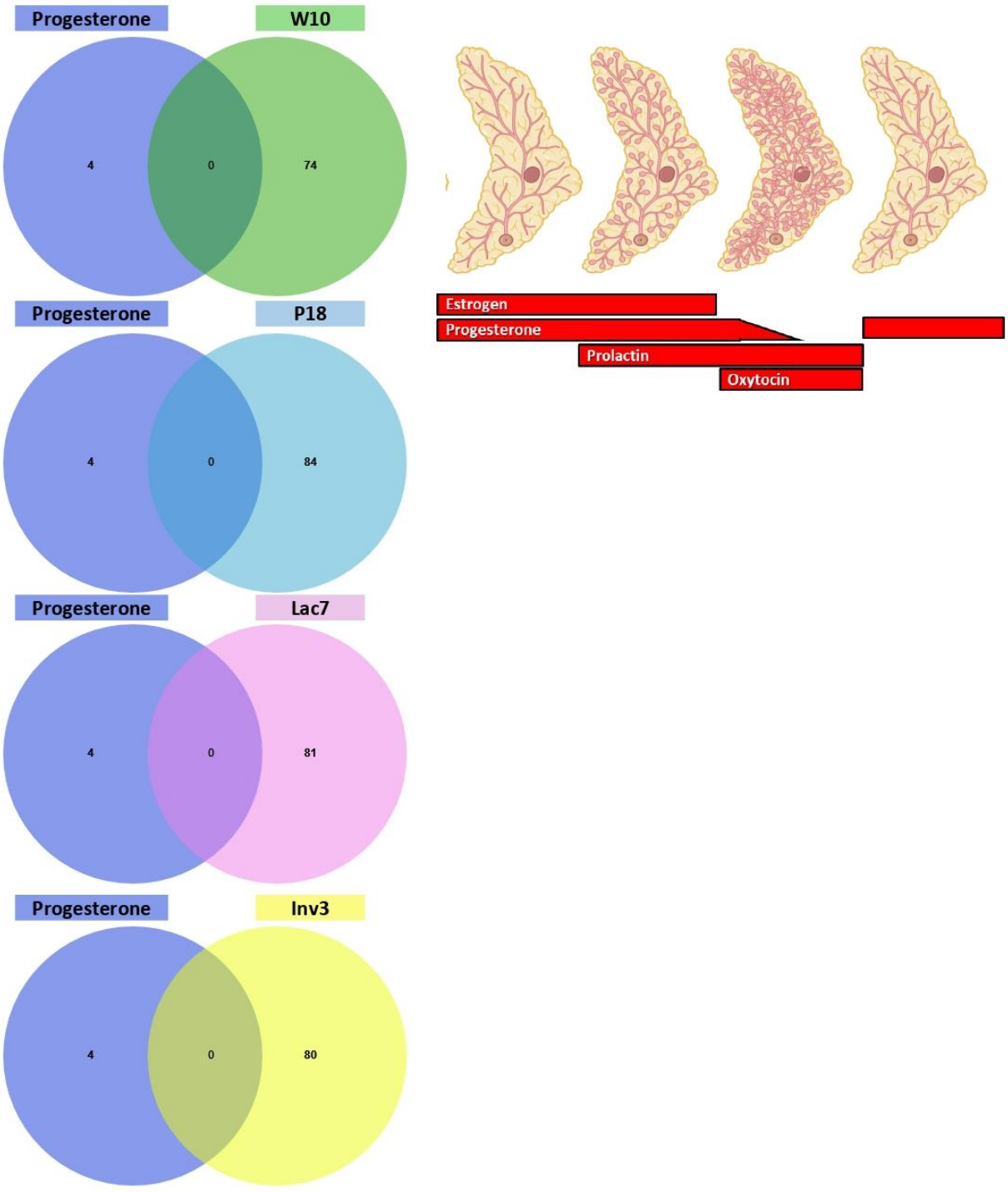
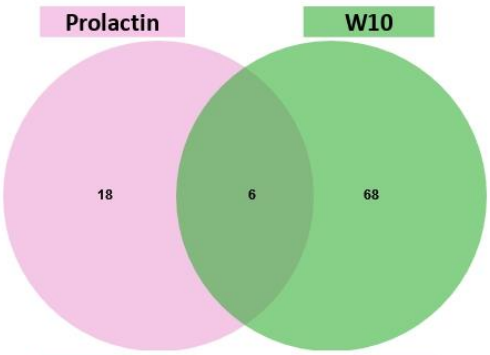
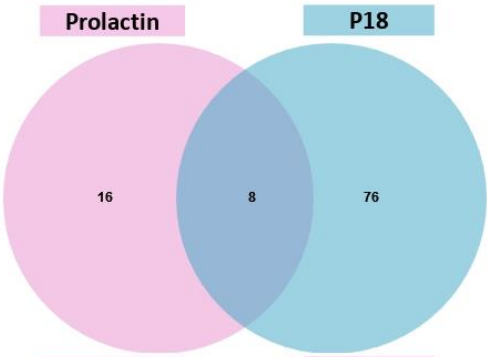
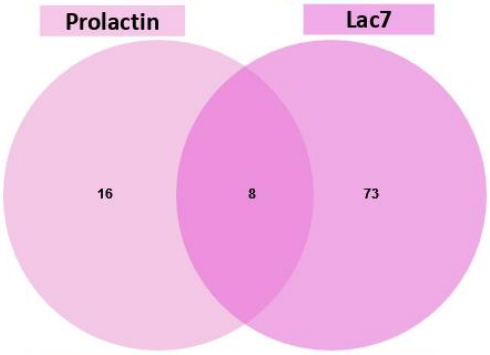
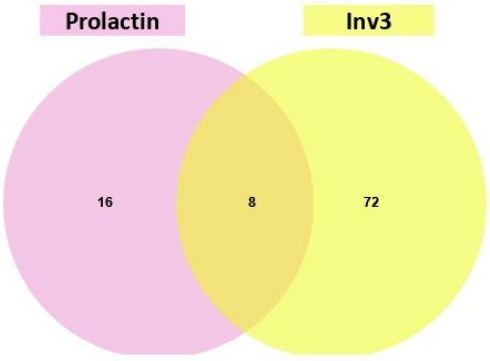


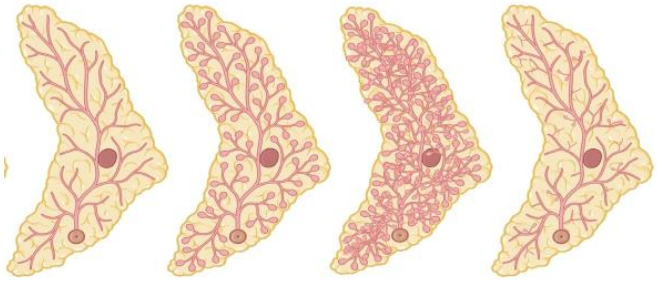
Figure 9: Comparison between miRNA expressed at each stage of development and miRNA modulated by progesterone. The Venn diagram shows that none of miRNAs that are modulated by progesterone are expressed at the four stages of development studied (≥.500 reads).

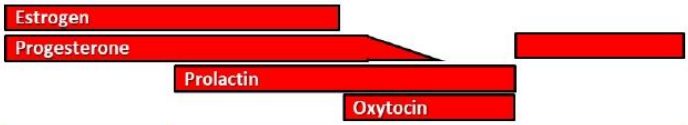






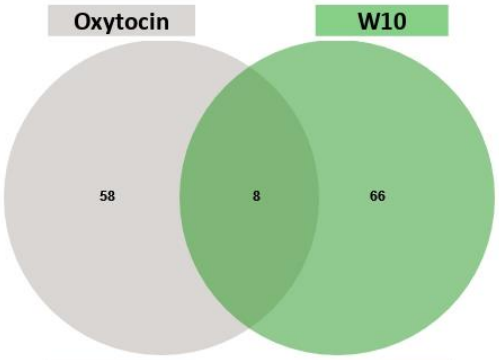


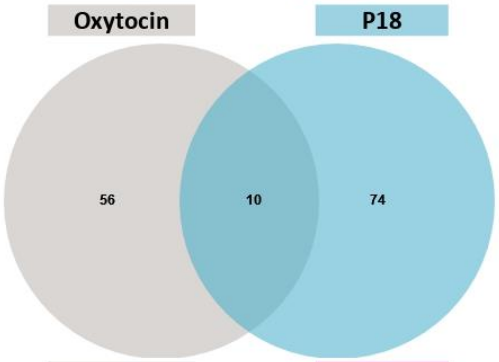


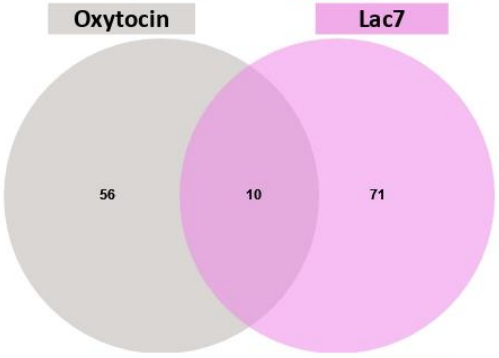


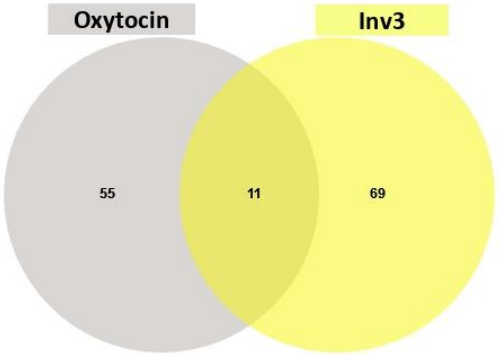
W10	P18	Lac7	Inv3
miR-30c-5p	miR-30c-5p	miR-30c-5p	miR-30c-5p
miR-16-5p	miR-16-5p	miR-16-5p	miR-16-5p
miR-181a-5p	miR-181a-5p	miR-181a-5p	miR-181a-5p
miR-125b-5p	miR-125b-5p	miR-125b-5p	miR-125b-5p
let-7b-5p	let-7b-5p	let-7b-5p	let-7b-5p
let-7d-5p	let-7d-5p	let-7d-5p	let-7d-5p
	miR-148a-3p	miR-148a-3p	miR-148a-3p
	miR-26b-5p	miR-26b-5p	miR-26b-5p

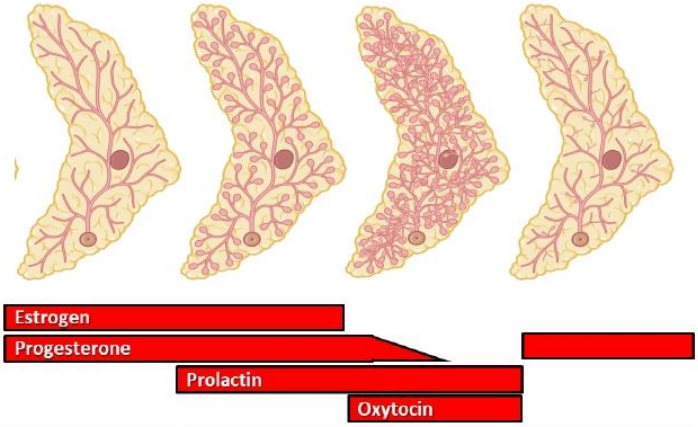
Figure 10: Comparison between miRNA expressed at each stage of development and miRNA modulated by prolactin. The Venn diagram shows that between 6-8 miRNAs that are modulated by prolactin are expressed at the four stages of development studied (≥.500 reads). The table shows the list of miRNA that were up-regulated (green) and down-regulated (red) by prolactin at each stage of development.











W10	P18	Lac7	Inv3
miR-125a-5p	miR-125a-5p	miR-125a-5p	miR-125a-5p
miR-200a-3p	miR-200a-3p	miR-200a-3p	miR-200a-3p
miR-30e-5p	miR-30e-5p	miR-30e-5p	miR-30e-5p
miR-192-5p	miR-192-5p	miR-192-5p	miR-192-5p
let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p
	miR-141-3p	miR-141-3p	miR-141-3p
	miR-141-5p	miR-141-5p	miR-141-5p
	miR-148a-3p	miR-148a-3p	miR-148a-3p
	miR-26b-5p	miR-26b-5p	miR-26b-5p
		miR-32-5p	
miR-92a-3p	miR-92a-3p		
miR-27a-3p			miR-27a-3p
miR-29b-3p			miR-29b-3p

Figure 11: Comparison between miRNA expressed at each stage of development and miRNA modulated by oxytocin. The Venn diagram shows that between 8-11 miRNAs that are modulated by oxytocine are expressed at the four stages of development studied (≥.500 reads). The table shows the list of miRNA that were up-regulated (green) and down-regulated (red) by oxytocin at each stage of development.

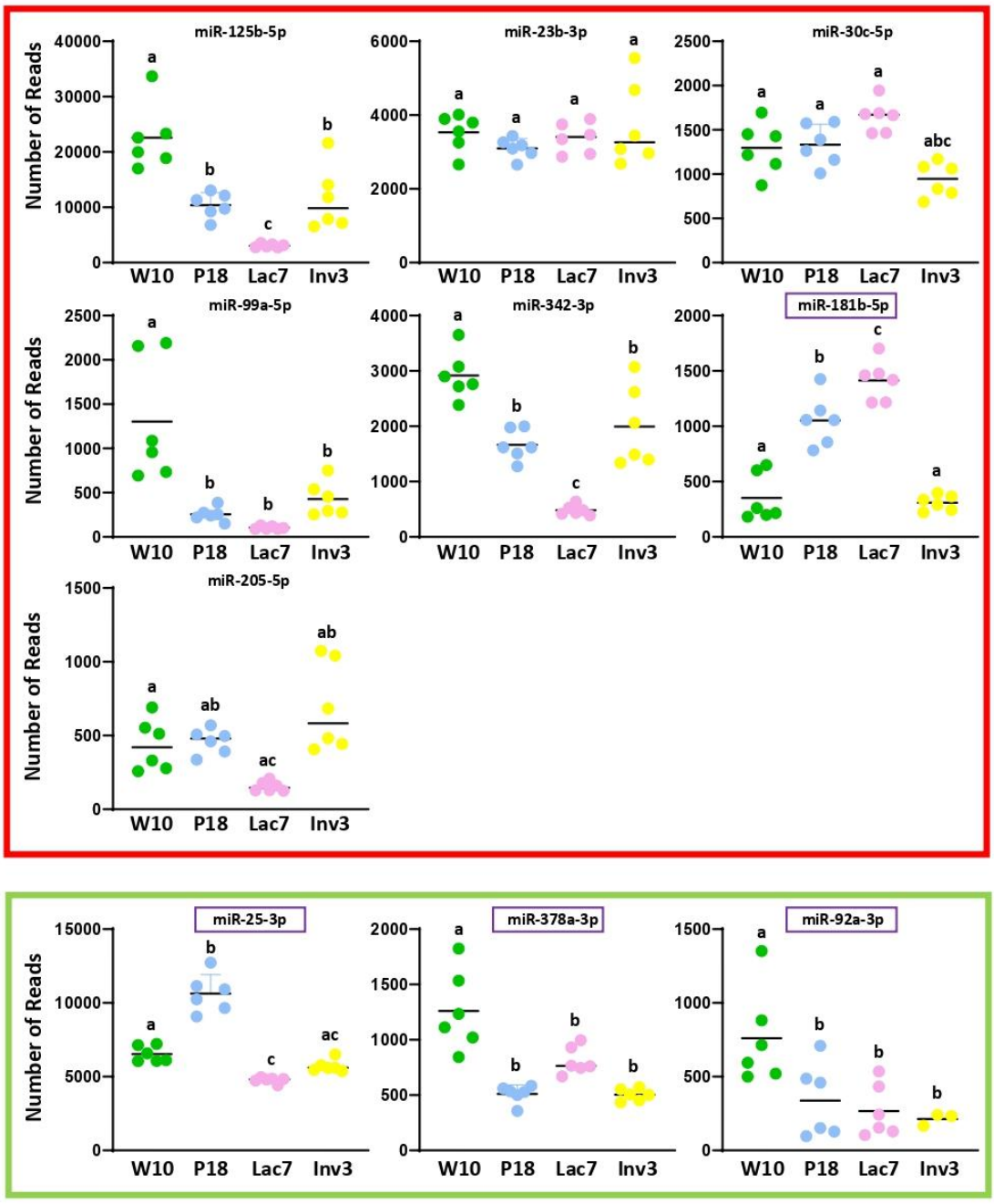


Figure 12: Levels of expression of miRNA potentially regulated by estradiol during mammary gland development. Small RNA were extracted from mammary glands using the mirVana™ miRNA Isolation (Thermo Fisher) and sequenced (Illumina NovaSeq6000). 6 animals were used per group (N=6). The red and the green squares indicate miRNA that were down- and up-regulated by estradiol *in vitro*, respectively. Purple squares indicate miRNAs whose expression correlates with estradiol levels *in vivo.* For all graphs, similar letters indicate no significant difference between the groups, while different letters indicate p≤0,05 as analyzed by an one-way ANOVA followed by a Tukey's multiple comparisons test.

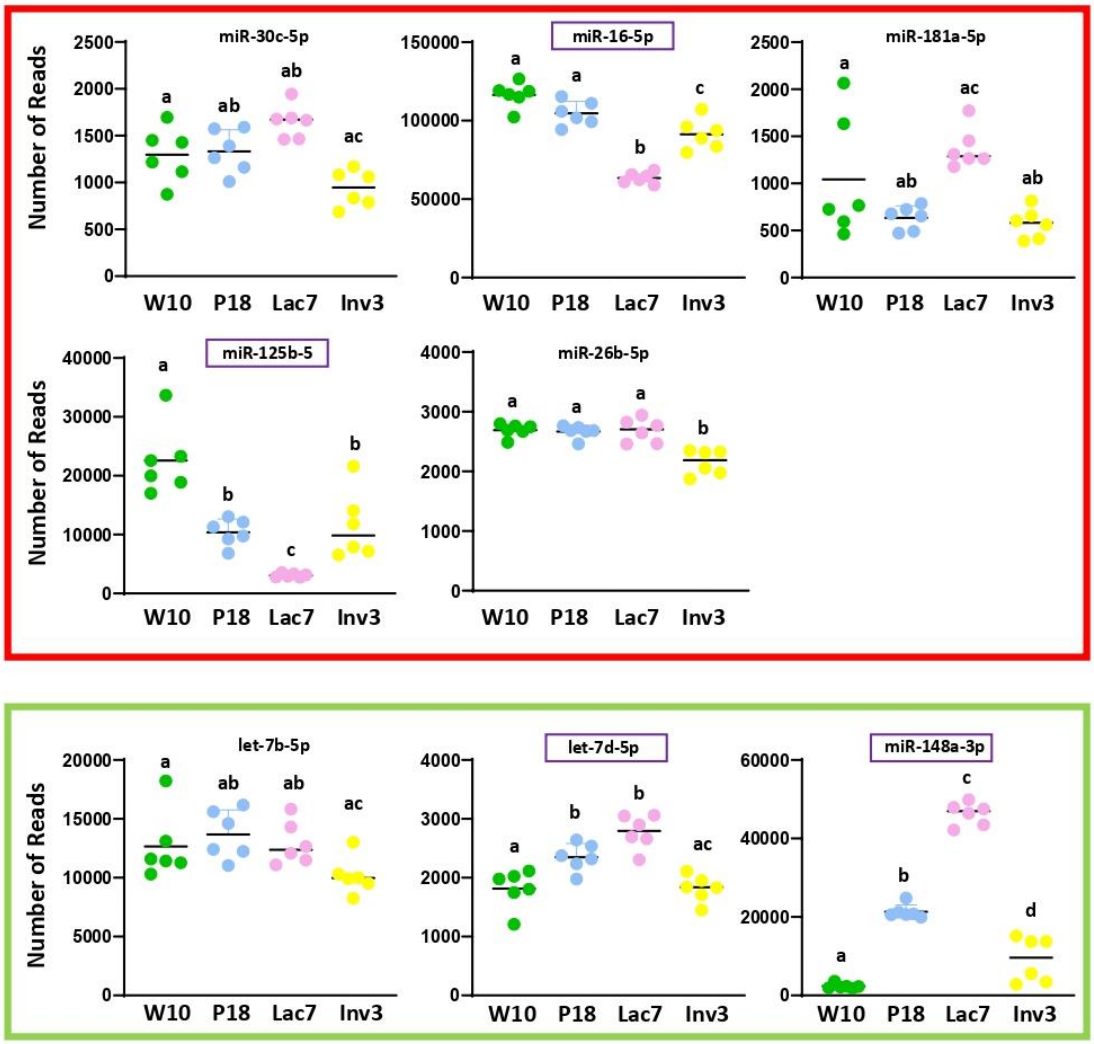


Figure 13: Levels of expression of miRNA potentially regulated by prolactin during mammary gland development. Small RNA were extracted from mammary glands using the mirVana™ miRNA Isolation kit (Thermo Fisher) and sequenced (Illumina NovaSeq6000). 6 animals were used per group (N=6). The red and the green squares indicate miRNA that were down- and up-regulated by prolactine *in vitro*, respectively. Purple squares indicate miRNAs whose expression correlates with prolactin levels *in vivo*. For all graphs, similar letters indicate no significant difference between the groups, while different letters indicate p≤0,05 as analyzed by an one-way ANOVA followed by a Tukey's multiple comparisons test.

levels of expression vary greatly between stages, especially between adult and lactating mice. As expected, differentially expressed miRNA are linked to processes and pathways related with remodeling and hormonal regulation. Surprisingly, a limited number of miRNAs were regulated by exposure to hormones *in vitro*, especially for progesterone. Nevertheless, by comparing *in vivo* and *in vitro* results, and referring to relative hormonal levels at the different stages, we were able to identify 16 miRNAs likely regulated by hormones in a stage-specific manner.

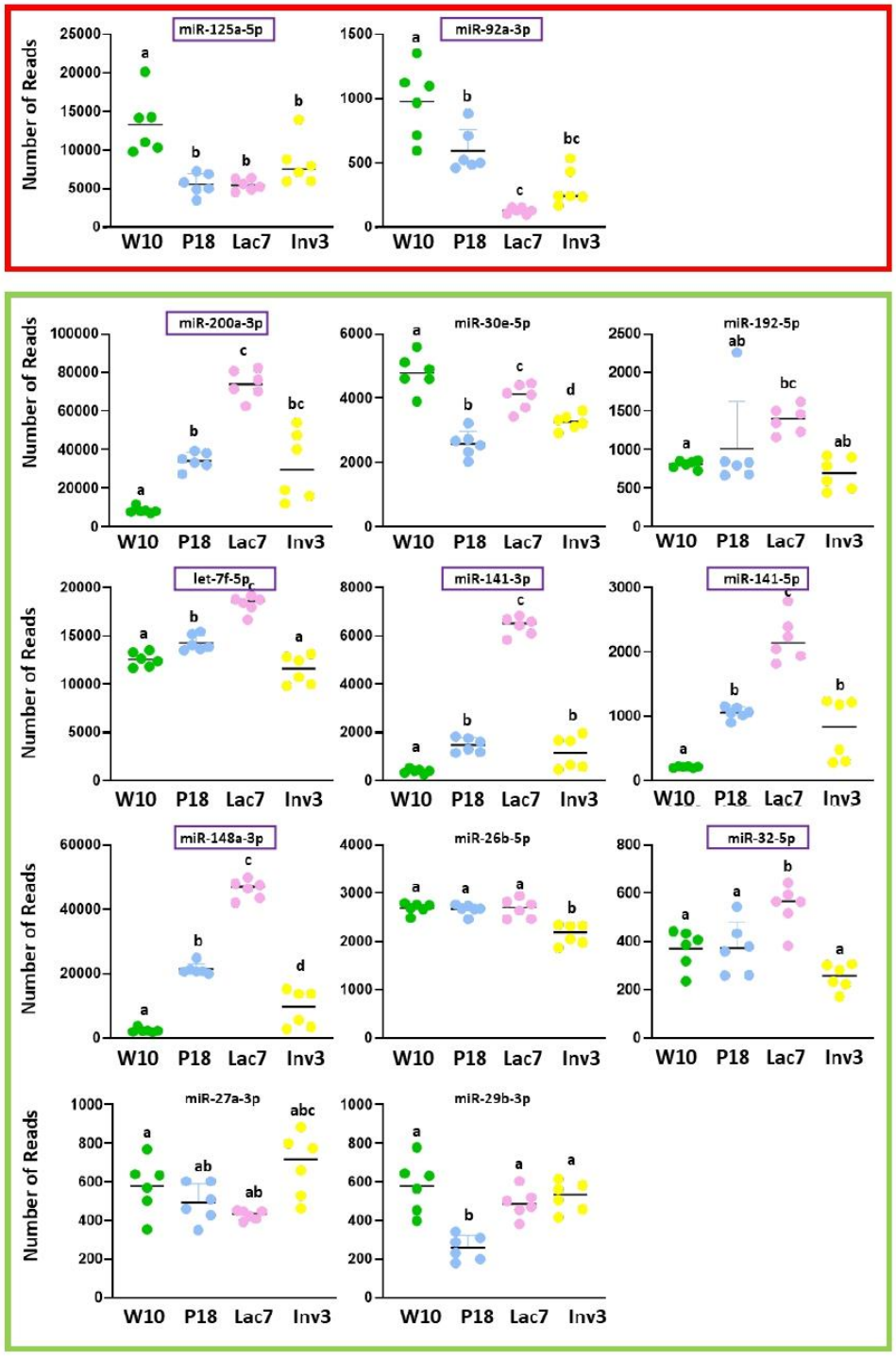


Figure 14: Levels of expression of miRNA potentially regulated by oxytocin during mammary gland development. Small RNA were extracted from mammary glands using the mirVana™ miRNA Isolation kit (Thermo Fisher) and sequenced (Illumina NovaSeq6000). 6 animals were used per group (N=6). The red and the green squares indicate miRNA that were down- and up-regulated by oxytocin *in vitro*, respectively. Purple squares indicate miRNAs whose expression correlates with prolactin levels *in vivo*. For all graphs, similar letters indicate no significant difference between the groups, while different letters indicate p≤0,05 as analyzed by an one-way ANOVA followed by a Tukey's multiple comparisons test.

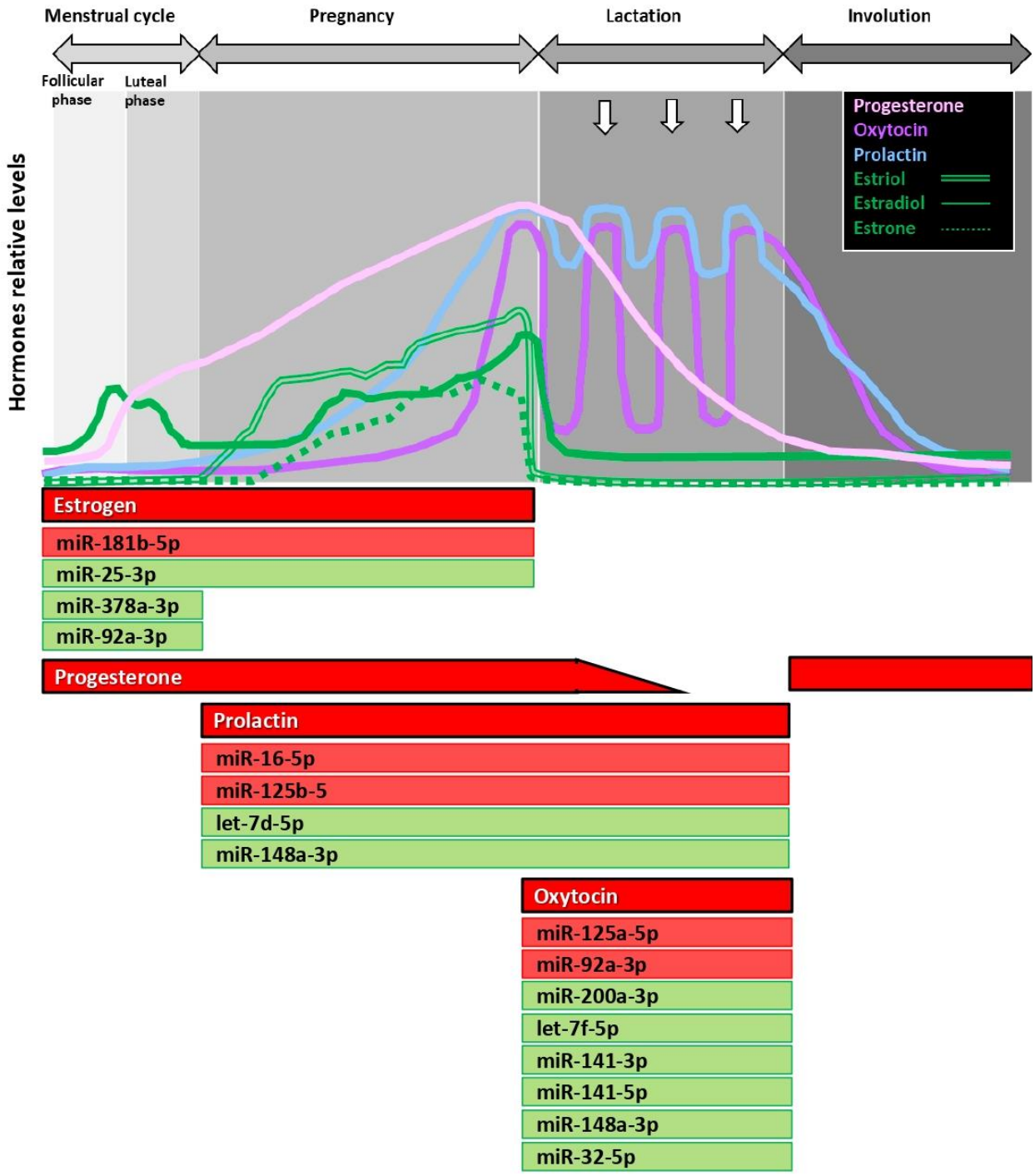


Figure 15: miRNA likely regulated by hormones at specific stages of mammary gland development. Top panel) Schematic illustration of the relative levels of key hormones involved in mammary gland development at the different stages. **Bottom panel)** miRNAs likely to be regulated by hormones at each stage of development, based on *in vivo* and *in vitro* results of the current study.

2.6.1 Limitations to take into consideration regarding the experimental design

Although our results bring interesting insight regarding the role of miRNA in mammary gland development, there are some limitations that should be considered when analyzing the data. First, our results represent the change in miRNA in the entire gland, as whole homogenates were done. Thereby, changes observed can be associated with change in miRNA expression in the epithelium, in the stroma, or in both compartments. Interestingly, data from Avril-Sassen and co-workers suggests that most miRNA differentially expressed in mammary gland homogenates are associated with mammary epithelium-driven events [13]. Thus, changes in most miRNAs detected in our analysis likely originate from the epithelium, even though the relative proportion of the stroma and epithelium varies between stages. Further analyses are needed to better understand the compartment-specific role and regulation of miRNAs in the mammary gland. Second, we limited ourselves to 4 developmental stages. The same above-mentioned study demonstrated that miRNA expression is similar from 6 weeks of age to early pregnancy, as for lactation and early stages of involution (between 12-48h after weaning) [13]. Based on these results, we chose to use adult (W10), pregnancy day 18 (P18), lactation day 7 (Lac), and involution day 3 (Inv) in our study to 1) optimize the distinction between miRNAs expressed at each stage, and 2) to get different hormonal and stage profiles. Finally, we used mouse tissues to evaluate stage-specific miRNA expression, but a human breast cancer cell line for the hormonal treatments. Tissues are composed of many different types of cells, each potentially expressing different miRNAs, while the *in vitro* study reports the response of only one cell type to the treatment. This could explain, in part, why a fewer number of miRNAs were identified upon the cell treatments. Part of the discrepancy can also come from the fact that T47-D cells are cancerous cells. However, they represent the luminal subtype of breast cancer and are not considered as aggressive. More importantly, they express the hormonal receptors, as oppose of many non-tumorigenic cell lines, which was crucial for our experiment. In addition, some interspecies differences could be present in miRNAs regulation. However, it has been demonstrated that there is a high level of conservation between human and mice miRNAs. In a study looking at conserved miRNAs between 51 vertebrates using BLAST, bovine and murine miRNAs showed similar levels of conservation, and were the closest to humans [42] [18]. Similarly, comparing mammospheres made from tissues from 6 mammals (cow, human, pig, horse, dog and rat), out of the 101 that were annotated, 86 were common to all 6 species [43], suggesting a good level of interspecies conservation. For this reason, in our study, the miRNAs commonly identified in mice and humans are likely to play a similar role within the mammary gland.

2.6.2 The number of miRNAs expressed and their expression levels vary between stages in a pattern consistent with mammary gland development

When miRNAs were sequenced from mammary glands of mice at key stages of development, we found that 70-80% of miRNA were commonly expressed at all stages analyzed, while 12-18% were expressed at only one stage. These percentages were similar whether we include all miRNAs detected (>1 read per million) or the ones though to be functionally relevant (>500 reads per million) [41]. Interestingly, when analyzing the miRNAs expressed, especially those specific to only one stage, there was an apparent inverse correlation between the number of miRNAs expressed and the remodeling and functional activity thought to happen at that stage specifically. Indeed, the highest number of total and uniquely expressed miRNA was found in adult mice, when the mammary gland is relatively quiescent, followed by pregnancy, when the gland is undergoing alveologenesis, then lactation, the functional stage, and involution. The lowest number of uniquely expressed miRNA were found during involution, when the gland experiences an intensive phase of apoptosis and remodeling, including regression of the alveoli and the re-expansion of the fat pad. A similar pattern was found in dairy goats, with about 80% of miRNA being commonly expressed between dry period (adult), late pregnancy and late lactation, with the highest number of stage-specific, highly expressed miRNA found in the dry period [18]. In cows, the lowest number of miRNA expressed in the milk fat layer, which is thought to be the closest representation of mammary gland tissue [44], was found in involution compared to lactation or galactopoiesis (established lactation) [45]. Considering that the main role of miRNAs is thought to be down regulation of mRNA levels, it suggests that many genes are repressed in adult mouse mammary glands but expressed at the other more active stages of development.

However, when analyzing expression levels between stages, the patterns were slightly different. 6 main clusters of expression could be found, with most of the miRNAs following clusters 1 and 3, which showed opposite tendencies. In cluster 1, an increased expression of the 93 miRNAs could be observed from adult mice up to lactation, followed by a decrease in involution. This pattern was confirmed when analyzing the levels of expression of miRNAs differentially expressed (DE) between stages, as out of the 30 DE miRNA, most were up-regulated between adult and pregnant mice, and between pregnant and lactating mice, while the majority were down regulated between lactation and involution. Similarly, a decreased number of miRNA and two-fold reduction of miRNA mean expression was observed between lactation and early involution in mice in a study using a bead-based flow-cytometric microarray analysis [13]. In contrast, the 135 miRNAs in cluster 3 showed a significant decrease in expression from adult to lactation, followed by an

increased expression at involution. For both clusters, most miRNA showed similar levels of expression between pregnancy and involution, although the expression was generally slightly lower at involution. Avril-Sassen and co-workers showed a decrease of expression at lactation, similar to our cluster 3, in their 3 largest clusters of expression, as well as an inverted cluster of expression with a peak of expression at lactation, similar to our cluster 1 [13]. These patterns of expression are consistent with the development of the mammary gland, as going from a relatively inactive stage (adult) gradually to a functional stage (lactation), and then returning to a more quiescent stage through the involution process requires activation and inhibition of specific signaling pathways.

2.6.3 miRNAs are differentially expressed especially during lactation

Lactation is a particularly important stage for the mammary gland as it is the functional stage of the gland, involving an important remodeling and differential expression of thousands of genes [46, 47]. In addition, it is a stage of importance for the offspring of all mammals, but especially for the cattle. As a result, many studies on miRNA have been conducted at this stage in this species raised in milk industries [14-16, 18, 21-23, 44, 45]. Le Guillou and collaborators showed that 123 miRNA are present in mouse and bovine mammary glands analyzed at lactation, and 24 out of the 30 expressed at high levels were common to both species, suggesting conservation of miRNA between these two species [23]. Among those miRNAs, they found 6 miRNAs (miR-126-5p, miR-16-5p, and members of the miR-200 family (miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p) that were present in both species at lactation, but not reported in other stages. In our study, although they were detectable at all stages, miR-141-3p and miR-200a-3p showed their highest expression level in lactation *in vivo* and were up-regulated by oxytocin *in vitro.* miR-200b-3p and miR-200c-3p expression was also detectable at all stages and peaked at lactation. In contrast, miR-16-5p was not only present in all stages but was down regulated at lactation and by prolactin, and miR-126-5p was not detected. Although these discrepancies could be explained by a different sampling time (lactation day 7 versus 12), a strain difference (C57BL/6 versus FVB/N) or parameters used for sequencing, miR-126-5p and miR-16-5p were not reported either to be specific or peaking at lactation in other studies, including in mice [13] [45] [14, 21]. Interestingly, using KEGG function annotations, Le Guillou and co-workers identified 83 biological processes targeted by miRNA highly expressed in lactation. Most of the pathways identified in the current study using the differentially expressed miRNA between pregnant and lactating mice, or between

lactating and involuting mice, were present on this list [23], and also in studies in the dairy goat [18, 48].

2.6.4 Several differentially expressed miRNA are hormonally regulated *in vitro*

Since mammary gland development is tightly regulated by hormones, we aimed to determine the relationship between changes in miRNA between stages of development and hormones associated with those stages. Using miRDB/v6.0 we first determined genes predicted to be targeted by DE miRNA between stages. As expected, many potential targets were associated with reproductive and metabolic hormones known to play a role in mammary land development (Figure 1), including receptors for estrogen, prolactin, oxytocin, insulin, thyroid and growth hormone, and many related factors. The number of predicted targets was the highest for estrogen, insulin-like and thyroid signaling. These results suggest that miRNA can regulate hormonal signaling within the mammary gland, which is in concordance with studies demonstrating that overexpression of specific miRNAs results in a decrease in transcript or protein levels of estrogen receptor (ER)α and progesterone receptor, as well as signaling and the expression of genes regulated by these receptors [49-52]. However, using this approach, we could only identify genes likely targeted by miRNAs, but not miRNAs whose expression could be modulated by hormones. Thus, using an *in vitro* approach, we analyzed the 4 reproductive hormones that are known to play crucial roles in mammary gland development, and found that the expression of 38, 4, 24 and 66 miRNA was significantly changed by estradiol, progesterone, prolactin and oxytocin, respectively. Within this list, a few miRNAs were already identified as estradiol regulated, such as miR-181a, miR-92a, miR-30b, and miR-23b, which were also regulated by estradiol in our experiments using T47-D cells [49, 52], or expressed in ERα-positive T47-D cells, such as miR-23, miR125b and miR-30c [53].

The effects of progesterone on miRNA expression in breast cells have not been examined by many studies. One study in mouse mammary epithelial cells demonstrated that miR-129-2 targets the progesterone receptor (PR) and is up-regulated in response to progesterone [51]. In T47-D cells treated for 6 hours with medroxyprogesterone acetate (MPA), a synthetic progesterone molecule, 28 miRNAs were differentially expressed, 20 being down regulated and 8 up-regulated [28]. Although for many miRNAs the trends were similar after 24 hours of treatment, none reached statistical significance, suggesting a transient effect. We found only 4 miRNAs that were significantly changed ($p \leq 0.05$) upon progesterone treatment, although 179 showed a log2 fold change above 1.5, including some that were identified by Cochrane and collaborators [28].

Whether the discrepancies between our results and their study is due to the difference in molecules used or time of exposure remains to be determined.

Similarly, only a few studies have analyzed the links between prolactin or oxytocin with miRNAs. In a recent article, miR-148a and miR-26a were up-regulated, while miR-320 was down-regulated in MCF10A cells, a non-tumorigenic human breast cell line, upon treatment with oxytocin [54]. In our study, 66 miRNAs were affected by oxytocin, including miR-148a and miR-26a that were also up-regulated, and miR-320 (320a-3p, 320b, 320c) that was down regulated. Interestingly, the authors showed that the expression of miR-148a and miR-320 was also modified in extracellular vesicles secreted by MCF10A cells upon the treatment, as well as in human milk from mothers who received oxytocin during delivery, further supporting an important role of these miRNAs in oxytocin-induced signaling in the mammary gland. In addition, a few other miRNAs that were significantly expressed upon oxytocin treatment in our study, were also affected by an oxytocin treatment in pregnant women [54]. In another study, bovine mammary epithelial cells (BMECs) were exposed to a mixture of lactogenic hormones (dexamethasone, insulin and prolactin) for 6 days, and miRNA expression was analyzed in both the media and the cells [55]. The treatment induced a down-regulation of miR-21-5p, miR-25, miR-26a, miR-223, and miR-320a in the cells, a down-regulation of miR-155, miR-182, miR-200c, and miR-339a in the BMEC culture medium, and an up-regulation of miR-148a in the BMEC culture medium [55]. Finally, miR-135b expression was shown to be expressed at early lactation in goats, gradually increasing until the end of lactation; its overexpression in epithelial cells was associated with decreased expression of β-casein and fat droplet formulation, two events associated with milk production [56]. In addition, exposure to prolactin reduces its expression, supporting a role of miRNA in prolactin-induced milk production [56]. In our study, miR-135b expression was low at all stages of development studied *in vivo*, and was only slightly decreased (not significant) upon prolactin treatment. This could be related to a species discrepancy, or a difference in the treatment as we used a lower dose *in vitro*.

2.7 Conclusion

By combining the patterns of expression of miRNA during mammary development and the miRNAs with expression shown to be regulated by hormones, we identified 16 mammary gland miRNAs whose expression is likely regulated by circulating hormones. While some of them had already been identified to be important in mammary gland development, in breast cancer, or regulated by steroid hormones, our study suggests that there is a relationship with their

expression and main hormones involved in mammary gland development (Figure 15). Future studies will further examine this role more in detail.

2.8 Acknowledgements

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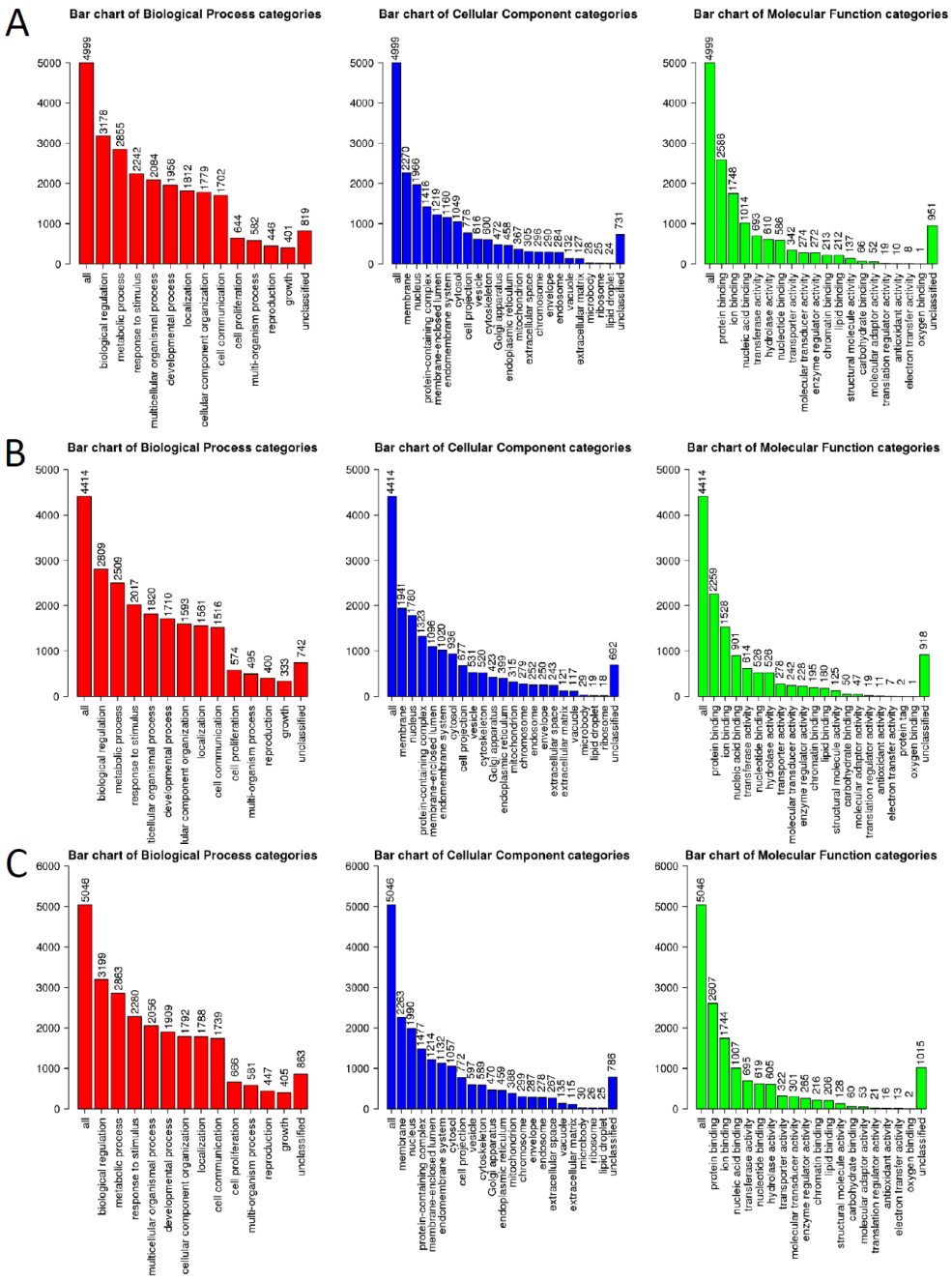
2.9 Supplementary material

As Tables 2-13 are a large amount of pages, those tables are available online at the following link: https://acrobat.adobe.com/id/urn:aaid:sc:US:95c497da-cda9-4529-bb83-c99d4bf99e60



Supplementary Figure 1: Biological processes, cellular components and molecular fractions predicted to be modified by miRNA between the different stages of mammary gland development. Using the WEB-based GEne SeT AnaLysis Toolkit (Wang, 2013), functional predictions were made based on change in mammary gland miRNA levels between virgin adult (W10) and pregnant mice (P18) (A), P18 and lactating mice (Lac7) (B) and Lac7 and involuting mice (Inv3) (C). To do so, a list of target genes was retrieved (containing 4999, 4414 and 5046 unique entrezgene ID, respectively), and submitted to a GO enrichment analysis. Target gene were collected from miRDB/v6.0 (Chen, 2020), that is using MirTarget/V4 (Liu, 2019) for predictions and miRBase/22 for miRNAs (Kozomara, 2019). Functional analyses were clustered by biological processes (red charts), cellular components (blue charts) and molecular function (green charts) categories. The number of unique entrezgene ID in each category is indicated on top of each bar.

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2.11 Link with second article, 'Identification of hormone-linked microRNA involved in regulation of Cx43 in the mammary gland'

This article found that miRNA vary in expression across different stages of mammary gland development, collectively as clusters, and individually. In addition, the miRNA regulated upon estradiol, progesterone, prolactin and oxytocin exposure were identified in separate experiments in T-47D cells. It was also found that their expression is predicted to be linked with numerous pathways regulated by hormones, or associated with transition between stages. These studies allowed us to identify hormone-associated miRNA potentially regulating mammary gland development. However, regulation of Cx43 expression by those miRNA, and how they may contribute to gap junction intercellular communication throughout the stages, was still unclear by this point. Additional analyses were hence needed a) to predict which miRNA might regulate Cx43 based on consensus sequences; b) to link miRNA expression with Cx43 mRNA expression; c) to

predict how hormones affect Cx43 mRNA; and d) confirm whether or not Cx43 mRNA levels are modulated by miRNA.

CHAPTER 3 : RESULTS PART 2

3.1 Article #2 introductory section

The objectives of this article are the following: 1) Predict miRNA candidates for Cx43 regulation using online databases; 2) Identify which candidates are both inversely correlated to Cx43 mRNA and associated with hormones*;* 3) Test the regulation of Cx43 mRNA by those same hormones; and 4) Confirm the effect of select candidates for Cx43 regulation on its mRNA levels, based on results of the previous objectives.

The hypothesis of this article is the following: some *in silico*-predicted candidates are inversely correlated to Cx43 and linked with hormones, suggesting miRNA regulation of its mRNA levels, in a hormone-dependent manner. We also hypothesize that one or more of those candidates functionally suppress Cx43 mRNA, confirming regulation of its expression.

To accomplish the objectives of this article, online databases were searched for miRNA candidates for Cx43 regulation. The expression of each miRNA *in vivo* and *in vitro* upon exposure to hormones was analyzed to identify candidates for Cx43 regulation that are inversely correlated with its mRNA levels and associated with hormones. To assess the effect of hormones on Cx43 mRNA, T-47D cells were exposed to estradiol, progesterone, prolactin and oxytocin for 0 to 8 h. Then, total RNA was extracted and reverse transcribed, and Cx43 cDNA was quantified using qPCR. To confirm the effect of miRNA on Cx43 expression, miRNA mimics and inhibitors, which increase and decrease a specific miRNA respectively, will be transfected in T-47D cells. Then, change in Cx43 mRNA will be assessed as above.

Identification of hormone-linked microRNA involved in regulation of Cx43 in the mammary gland

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3.2 Abstract

The mammary gland is a unique organ as most of its development happens postnatally, mainly through the action of hormones. It also requires communication between cells, including through gap junctions. Gap junctions are intercellular channels allowing direct communication between adjacent cells, and made of a family of protein named connexins (Cxs). Dysregulation of connexin 43 (Cx43) has been associated with developmental and functional defects in the mammary gland, as well as with breast cancer. However, mechanisms of Cx43 regulation are complex and minimally understood. MicroRNA (miRNA) are short RNA molecules that post-transcriptionally regulate the expression of RNA, typically through complementary binding with the 3' untranslated region of mRNA. Their role in regulation of Cxs within the mammary gland is poorly understood. Our study aimed to identify miRNA regulated by key hormones involved in mammary gland development that regulate Cx43 expression. We first used an *in silico* approach to identify miRNA that can regulate Cx43 based on their consensus binding sequences. Then, their levels of expression were analysed *in vivo* in mammary glands sampled from adult, pregnant, lactating and involuting mice. Analyses found that several bioinformatics-predicted candidates for Cx43 regulation, such as miR-200a-3p, miR-30c-5p and miR-101-3p were inversely correlated to Cx43 expression, suggesting potential negative regulation of its mRNA levels. Using breast cells, we then demonstrated that the expression of some miRNA predicted to be regulators of Cx43 miRNA was modified upon exposure to estradiol, progesterone, prolactin, or oxytocin. In parallel, we showed that Cx43 expression is down-regulated by prolactin and up-regulated by estradiol. Results suggest that miRNA potentially interact with and regulate Cx43, contributing to proper Cx43 expression and mammary development and function.

3.3 Introduction

The mammary gland comprises a ramified, secretory epithelium surrounded by a supportive stroma. At birth, the epithelium is rudimentary; then, a surge of hormones at puberty stimulates ductal elongation and ramification (Macias and Hinck 2012). In adults, the epithelium remains mostly quiescent, experiencing relatively small cycles of remodeling following the estrus cycles. During pregnancy, the epithelium undergoes massive expansion, leading to the formation of alveoli, milk-secreting structures that become functional during lactation (Biswas et al. 2022). At weaning, the stagnation of milk and reduced stimulation induce involution, whereby the mammary gland regresses via apoptosis, returning to a pre-pregnancy level of complexity (Stein, Salomonis and Gusterson 2007).

Epithelial proliferation, differentiation and function are tightly regulated by the ovarian steroid hormones estradiol and progesterone, as well as the pituitary peptide hormones prolactin and oxytocin (Macias and Hinck 2012; Lollivier et al. 2006). Estradiol stimulates ductal elongation mainly during puberty, but also participates in side-branching (Macias and Hinck 2012). Side-branching is regulated by progesterone, which during lactation also synergizes with prolactin to mediate alveologenesis, both through the receptor activator of NF-κB ligand (RANKL) and Wingless/Integrated (Wnt) pathways (Fernandez-Valdivia et al. 2008; Macias and Hinck 2012). Prolactin acts through the STAT5 pathway to increase lactation-associated proliferation and prompt milk component synthesis (Macias and Hinck 2012). Last, oxytocin elicits outer myoepithelial cell contraction, causing milk release from inner luminal cells of alveoli into the ducts leading to the nipple, enabling offspring nourishing (Haaksma, Schwartz and Tomasek 2011). Oxytocin also stimulates the release of prolactin, thus maintaining milk production (Kennett and McKee 2012).

The proper development and remodeling of the mammary gland also requires communication between luminal and myoepithelial cells forming the bilayered epithelium, as well as between the stroma and the epithelium. Notably, we have demonstrated that the expression of gap junction proteins connexins (Cxs) is stage-dependent and that they form a junctional nexus within the epithelium whose composition varies in a stage-specific manner (Dianati et al., 2017). Gap junctions arise via assembly of hemichannels from opposing cells, each formed by hexamers of transmembrane Cxs (Stewart, Simek and Laird 2015). They allow direct passage of carbohydrates (Locke et al. 2004; Wang and Veenstra 1997), nucleotides (Goldberg et al. 2002), nucleic acids (Valiunas et al. 2005; Zong et al. 2016), second messengers (Bedner et al. 2006; Hernandez et al. 2007; Bevans et al. 1998, Sàez et al. 1989), and amino acids (Kawanokuchi et al. 2006) between cells, coordinating, among others, growth, division and differentiation (Goodenough and Paul 2009; Shaw et al. 2007; Zefferino et al. 2019; Delmar et al. 2018). As a result, inhibition of Cxs or gap junction intercellular communication (GJIC) has been associated with developmental defects and diseases, including breast cancer (Unal et al. 2022). Notably, our previous work showed that a mutation within the gene coding for Cx43 results in a decrease of its expression, resulting in a delayed epithelium development and lactation incapacities in mice (Plante and Laird 2008; Plante et al. 2010). We also demonstrated that this mutation delayed the onset of palpable tumors in the breast, but induces hyperplasia and increases metastasis to the lungs (Plante et al. 2011). In addition, Cx43 was dysregulated in a subtype-specific manner in breast cancer (Busby, Hallett and Plante 2018). Together, these results suggest that Cx43 plays

a crucial role in mammary gland development, and thus, understanding Cx43 regulation is crucial to better understand its role in breast cancer.

Mechanisms of Cx43 regulation include DNA methylation, histone acetylation, transcription factors, translation variants, post-translational modifications, and protein-protein interactions (M. Oyamada, Takebe and Y. Oyamada 2013). Despite extensive knowledge on GJs, Cx43 regulation by microRNA (miRNA) and its link with hormone pathways are poorly known. miRNA are 19 to 25 nucleotide non-coding RNA that downregulate mRNA expression via 3' UTR or translation start site complementary binding, which results in transcript cleavage or translation blockage, respectively (Reichholf et al. 2019). A few studies have demonstrated a role of miRNA in Cx43 expression. As an example, Cx43 regulation by miR-206 is necessary to electrical coupling of heart cells (Jin et al. 2019) and is implicated in bone (Inose et al. 2009) and muscle development (Anderson et al. 2006). It also has been suggested to be involved in mammary gland (Ming et al. 2015; Fu et al. 2015) and ovary (Yu et al. 2020) development, though its mechanistic role in these tissues is less studied. In breast cells, Ming et al. carried out transfections with different miRNA candidates for Cx43 regulation, and found that miR-1, miR-206, miR-200a, miR-381, miR-23a, miR-23b and miR-186 functionally suppressed its expression (Ming et al. 2015). Moreover, miR-200a-3p was determined as a direct binder of Cx43 mRNA via its 3' untranslated region (3' UTR) (Ming et al. 2015).

The effects of hormone exposures on miRNA expression have also been characterized in breast cancer cells, but only in limited studies. For example, MCF-7 and ZR-75.1 cells were exposed to estrogen, and it was found that 172 miRNA were modulated by the treatment (Ferraro et al. 2012). Using a different treatment time but same concentration of estrogen, Bhat-Nakshatri and co-workers identified 28 miRNA differentially expressed upon estrogen exposure in MCF-7 cells (Bhat-Nakshatri et al. 2009). In another study, 28 miRNA displayed a fold change above 1.5, and had levels significantly altered upon exposure to progestin in T-47D cells (Cochrane et al. 2012). A further study investigating the effects of prolactin on lipogenic miRNA found that 4 miRNA were elevated in response to prolactin, beginning at 2 µg/mL and most pronounced at 10 µg/mL in goat mammary gland epithelial cells (Lin et al. 2013). Lastly, miRNA can also participate in more complex feedback pathways. In MCF-7 cells, an estrogen receptor alpha (ERα) agonist was found to decrease expression of miR-206 (Adams et al. 2007). At the same time, it was shown that miR-206 directly targets ERα mRNA via its 3' UTR in HeLa cells, suggesting a possible double negative feedback loop (Adams et al. 2007). Together, these studies suggest possible hormone activation or deactivation of Cx43 expression through miRNA.

In this work, we aim to identify miRNA regulated by key hormones involved in mammary gland development that regulate Cx43 expression. To do so, we used a combination of *in silico, in vitro* and *in vitro* approaches.

3.4 Materials and methods

3.4.1 *In silico* analysis

TargetScan Human 8.0 and TargetScan Mouse 8.0 were searched for potential miRNA binding partners of Cx43 mRNA (GJA1 gene). The databases analyze complementarity between the 3' UTR of mRNA and various miRNA seed sequences, among other factors, in elucidating potential regulation of mRNA by miRNA (Lewis, Burge and Bartel 2005). 'Table of miRNA Binding Sites' was analyzed, and miRNA from 'Conserved among vertebrates', 'Conserved among mammals' and 'Poorly conserved but confidently annotated miRNA' lists were selected based on their total context score; decreased total context score represents increased potential magnitude of regulation (Agarwal et al. 2015). miRNA with a total context score above -0.20 were removed, as these miRNA likely have a lesser role to play in mRNA regulation.

3.4.2 Animals

Female C57BL/6 mice were purchased from Charles River Canada (St. Constant, Quebec, Canada). Mice were maintained under a constant photoperiod of 12h light:12h dark and received food and water ad libitum. All animal protocols used in this study were approved by the University Animal Care Committee (INRS-Armand-Frappier Santé Biotechnologie, Laval, Canada). Female mice were sacrificed using CO_2 followed by cardiac puncture, and the mammary glands were collected at the following time points: adult (W10), pregnancy day 18 (P18), lactation day 7 (Lac), and involution day 3 (Inv). For each developmental stage, 6 mice were sampled (N=6). The mammary gland pairs 4 and 5 (abdominal and inguinal) were flash-frozen in liquid nitrogen immediately after dissection.

3.4.3 *In vivo* miRNA isolation and sequencing

Frozen tissues were ground in liquid nitrogen. miRNA were extracted from the resulting powder using the *mir*Vana™ miRNA isolation kit following the manufacturer's instructions (Thermo Fisher Scientific, Mississauga, ON, Canada). The miRNA concentration was quantified with a Nanodrop

1000 (Thermo Fisher Scientific), while purity and integrity were assessed using the Agilent Small RNA kit (Agilent, Wilmington, DE, USA) and an Agilent bioanalyzer 2100. Data were analyzed using the Bioanalyzer 2100 Expert (version B.02.11.S1824 SR1) software; digital gel images and electropherograms of samples were validated prior to sequencing. miRNA libraries were prepared using a QIAseq miRNA stranded kit (Quiagen, Toronto, ON, Canada) and sequenced using an Illumina NextSeq 500 apparatus. The mapping against reference genome was done using QuickMirSEq and miRbase/22 (Kozomara et al, 2019; Zhao et al., 2017). Analyses of differently expressed miRNA and of clustering were performed using DESeq2/1.26.0, DEGReport and bigPint applications (Rutter et al., 2020; Stephens et al., 2017).

3.4.4 Cell culture

T-47D cells, a luminal breast cancer cell line originating from a 54-year old female patient, were obtained from American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI-1640) media without phenol red (Thermo Fisher, USA), supplemented with fetal bovine serum (FBS) (Thermo Fisher, USA) at 10% final concentration was used to grow the cells in a 37^{o}C and 5% CO_2 environment. Cells were plated and passaged before 90% confluency to maintain log-phase growth. Doubling time was calculated and the cells were not kept for more than 10 passages.

3.4.5 Hormonal exposures

At 60 to 70% confluency and 16 hours prior to hormonal exposure, media was replaced with media containing hormone-stripped FBS (Wisent Bioproducts, CAN) to eliminate effects of background hormone signaling. In a first set of experiments, cells were exposed to hormones based on physiological levels and on efficient treatment conditions found in literature (Ferraro et al. 2012, Cochrane et al. 2012, Bhat-Nakshatri et al. 2009, Cook et al. 2015). The following treatments were used: 8.7 nM (200 ng/mL) and 8 hours for prolactin (L4021, Sigma-Aldrich, USA), 10 nM and 24 hours for estradiol (E8875, Sigma-Aldrich, USA), 10 nM and 6 hours for progesterone (P0130, Sigma-Aldrich, USA) and 100 nM and 24 hours for oxytocin (O6379, Sigma-Aldrich, USA). These cells were used to evaluate miRNA expression. In a second set of experiments, cells were exposed to the same concentrations of hormones, but the exposure durations were from 0 to 8 hours to assess the effects of hormones on Cx43 regulation. All experiments included exposures to hormone dissolution vehicles, which included 0.1% ethanol

for estrogen and progesterone, 4 mM hydrochloric acid for prolactin and nuclease-free water for oxytocin, as well as untreated controls.

3.4.6 Western blot

After hormone exposure, media was removed, and cells were washed with Dulbecco's phosphate buffered saline (Thermo Fisher, USA). Protein extraction buffer (Tris 50 mM, NaCl 150 mM, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 1.25 M NaF, 1 M NaVO3, and Halt Protease and Phosphatase Cocktail Inhibitor (Thermo Fisher, USA)) was then used to lyse the cells inside the vessel using a cell scraper. Homogenates were centrifuged at 2500g for 10 minutes and supernatants were collected and kept at -80°C. Total proteins were measured using Thermo Fisher's Pierce BCA protein assay kit (USA). Polyacrylamide gels with densities of 10 or 12.5% (TGX Stain-Free FastCast Acrylamide kit) (Bio-Rad, USA) were used to separate proteins for Western blots. Total proteins were transferred from gels to PVDF membranes and visualized, using the Trans-blot Turbo Transfer and ChemiDoc MP imaging apparatuses respectively (Bio-Rad, USA). The membrane was subsequently treated with 3% bovine serum albumin (BSA) or 5% powdered milk diluted in TBS-Tween 0.1% as a blocking solution. Then, in accordance with the manufacturer's recommendations and earlier optimisation, primary antibodies diluted in 3% BSA or 5% powdered milk in TBS-Tween 0.1% were applied (Supplementary table 2). After washing the membranes three times for five minutes with TBS-Tween 0.1%, anti-mouse or anti-rabbit secondary antibodies coupled with horseradish peroxidase were applied. The membranes were then incubated with Clarity Western ECL Blotting Substrate (or Clarity Max) and afterwards visualized using the ChemiDoc MP imaging apparatus (Bio-Rad, USA). Imaged proteins were quantified and normalized to the membrane's total protein content using the ImageLab 6.0 program (Bio-Rad, USA).

3.4.7 *In vitro* miRNA sequencing

After hormonal treatment, the media was removed, cells were first washed with Dulbecco's phosphate buffered saline (Thermo Fisher, USA) and then detached by incubating at 37ºC with trypsin-EDTA 0.25% (Thermo Fisher, USA) for 7 to 10 minutes. Then, cells were pelleted by centrifuging for 5 minutes at 125g, and the supernatant was discarded. The MirVana miRNA Isolation Kit (Thermo Fisher, USA) was used according to instructions to isolate small RNA samples from cells. miRNA were quantified using a Nanodrop 1000 (Thermo Fisher, USA), while purity and integrity were assessed using the Agilent Small RNA kit and an Agilent 2100

bioanalyzer (Agilent, USA). Data were analyzed using the Bioanalyzer 2100 Expert (version B.02.11.S1824 SR1) software, and only samples with digital gel images and electropherograms showing minimal degradation were retained. Extracts were then processed for miRNA-seq analysis (Illumina platform). FASTQC v0.11.8 was used for reads quality assessment, while Cutadept was used for reads trimming. miRNA amount was calculated using MIRDEEP and COMPSRA software, and DESeq2 software was applied to calculate differential expression within each separate hormone experiment. Where applicable, the removeBatchEffect software from limma was used to correct miRNA quantity.

3.4.8 RT-qPCR

Upon treatment, the media was removed, cells were washed with DPBS and detached by incubating at 37^{o}C with trypsin-EDTA 0.25% for 7 to 10 minutes. Then, cells were pelleted by centrifuging for 5 minutes at 125 g and the supernatant was discarded. The Arum Total RNA Mini Kit (Bio-Rad, USA) was used according to instructions to isolate total RNA samples from the pelleted cells. Total RNA quality and concentration were verified using a nanodrop spectrophotometer (Thermo Fisher, USA), followed by the Bioanalyzer 2100 Expert (version B.02.11.S1824 SR1) software, and only samples with ARN Integrity Number (RIN) above 8.0 were used. RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA). qPCR was conducted using the CFX96 thermal cycler (Bio-Rad, USA) and Sso Advanced Universal SYBR Green Supermix (Bio-Rad, USA) with the following amplification conditions: 95^{o}C for 30 seconds, then 40 cycles at 95^{o}C for 10 seconds and 60^{o}C for 30 seconds. The expression of GJA1 was normalized to UXT, RPL13A and HPRT1 reference genes. The stability of these reference genes was validated for our set of samples using CFX software (Bio-Rad, USA). For each primers set (Supplementary table 1), the conditions were optimized to insure that amplification was within the linear range (Ma, Bell and Loker 2021).

3.4.9 miRNA mimic and inhibitor transfections

MirVana miRNA mimic (4464066) and Anti-miR miRNA inhibitor (AM17000) for hsa-miR-125b-5p, as well as mirVana miRNA Mimic Negative Control #1 (4464058) and Anti-miR miRNA inhibitor negative control #1 (AM17010), were purchased from Thermo Fisher (USA). Cells were transfected using the Lipofectamine RNAiMAX reagent (Thermo Fisher, USA) following the manufacturer instructions. Briefly, lipofectamine and mimic/inhibitor were diluted in Opti-MEM reduced serum media (Thermo Fisher, USA) at 10 nM and cells were exposed for 48 hours. When

applicable, cells were also co-treated with 8.69 nM prolactin or 10 nM estrogen for the final eight hours of transfection. Then, cells were harvested, and miRNA was isolated as described previously.

3.5 Results

3.5.1 Bioinformatics searching identifies potential miRNA binders of Cx43 mRNA

In order to predict miRNA regulators of Cx43 mRNA, the databases TargetScan Mouse 8.0 and TargetScan Human 8.0 were searched for its potential miRNA binding partners (McGeary et al. 2019) (Figure 1). In the human analysis, 8 miRNA candidates for Cx43 regulation were found to be conserved between vertebrates, 8 between mammals and 14 were poorly conserved but confidently annotated miRNA (Figure 1A), whereas in the mouse analysis, 14 miRNA were conserved between vertebrates, 10 between mammals and 21 were poorly conserved but confidently annotated miRNA (Figure 1B).

3.5.2 Identification of miRNA with inverse correlation in vivo to Cx43 mRNA expression

To identify miRNA expression across various stages of mammary gland development in the context of Cx43 regulation, we sequenced miRNA extracted from mouse mammary glands at stages where Cx43 expression changed most significantly based on our previous studies (Dianati et al., 2016). The expression of each of the Cx43 regulator candidates identified *in silico* (Figure 1) was analyzed to select miRNA candidates for Cx43 regulation based on an inverse correlation with its mRNA levels (Figure 2). In our previous study, Cx43 expression was decreased at lactation, and higher at W10, P18 and Inv (Dianati et al., 2016); miRNA with a high expression at lactation were thus considered as inversely correlated. Interestingly, out of 19 expressed (above 500 RPM) candidates (Figure 1), 6 miRNA were inversely correlated with a peak expression at lactation (Figure 2E, F, G, J, N, O), and 2 were inversely correlated but showing high expression at involution or W10 (Figure 2M, P). On the other hand, at 6 were positively correlated with Cx43 expression, showing the lowest levels at lactation (Figure 2B, I, L, Q, R, S), and 4 show lowest levels at lactation, but also at other stages (Figure 2C, D, H, K). No clear relationship was observed for some of those positively miRNA (Figure 2). In addition, 5, 7 and 7 of the miRNA were identified as candidates for Cx43 regulation in both mouse and human, in human, and in mouse databases, respectively.

TARGETSCAN HUMAN 8.0

Broadly conserved among vertebrates

miRNA	Total Context Score
miR-1-3p/206	-0.84
miR-19-3p	-0.65
miR-130-3p/301-3p/454-3p	-0.61
miR-101-3p.1	-0.41
miR-30-5p	-0.27
miR-17-5p/20-5p/93-5p/106-5p/519-3p	-0.26
miR-129-3p	-0.26
miR-218-5p	-0.21

Conserved among mammals

miRNA	Total Context Score
miR-185-5p	-0.28
miR-1306-5p	-0.26
miR-501-3p/502-3p	-0.24
miR-186-5p	-0.23
miR-744-5p	-0.21
miR-323-3p	-0.21
miR-382-3p	-0.2
miR-873-5p.2	-0.2

Poorly conserved, but confidently annotated

miRNA	Total Context Score
miR-1911-5p	-0.4
miR-520g-3p	-0.29
miR-519-3p	-0.27
miR-3681-5p	-0.24
miR-374b-3p	-0.24
miR-515-5p/519e-5p	-0.23
miR-3622a-5p	-0.23
miR-582-3p	-0.23
miR-500a-3p	-0.22
miR-511-3p	-0.21
miR-4428	-0.2
miR-4662-5p	-0.2
miR-361-3p	-0.2
miR-214-3p/3619-5p	-0.2

TARGETSCAN MOUSE 8.0

miRNA	Total Context Score
miR-1-3p/206-3p	-0.72
miR-101-3p.1	-0.44
miR-130-3p/301-3p	-0.43
miR-221-3p/222-3p	-0.41
miR-101a-3p.2/101b-3p.1/101b-3p.2	-0.38
miR-141-3p/200a-3p	-0.36
miR-199-3p	-0.35
miR-7-5p	-0.29
miR-125-5p/351-5p	-0.28
miR-34-5p/449-5p	-0.27
miR-19-3p	-0.27
miR-150-5p	-0.26
miR-31-5p	-0.23
miR-144-3p	-0.2

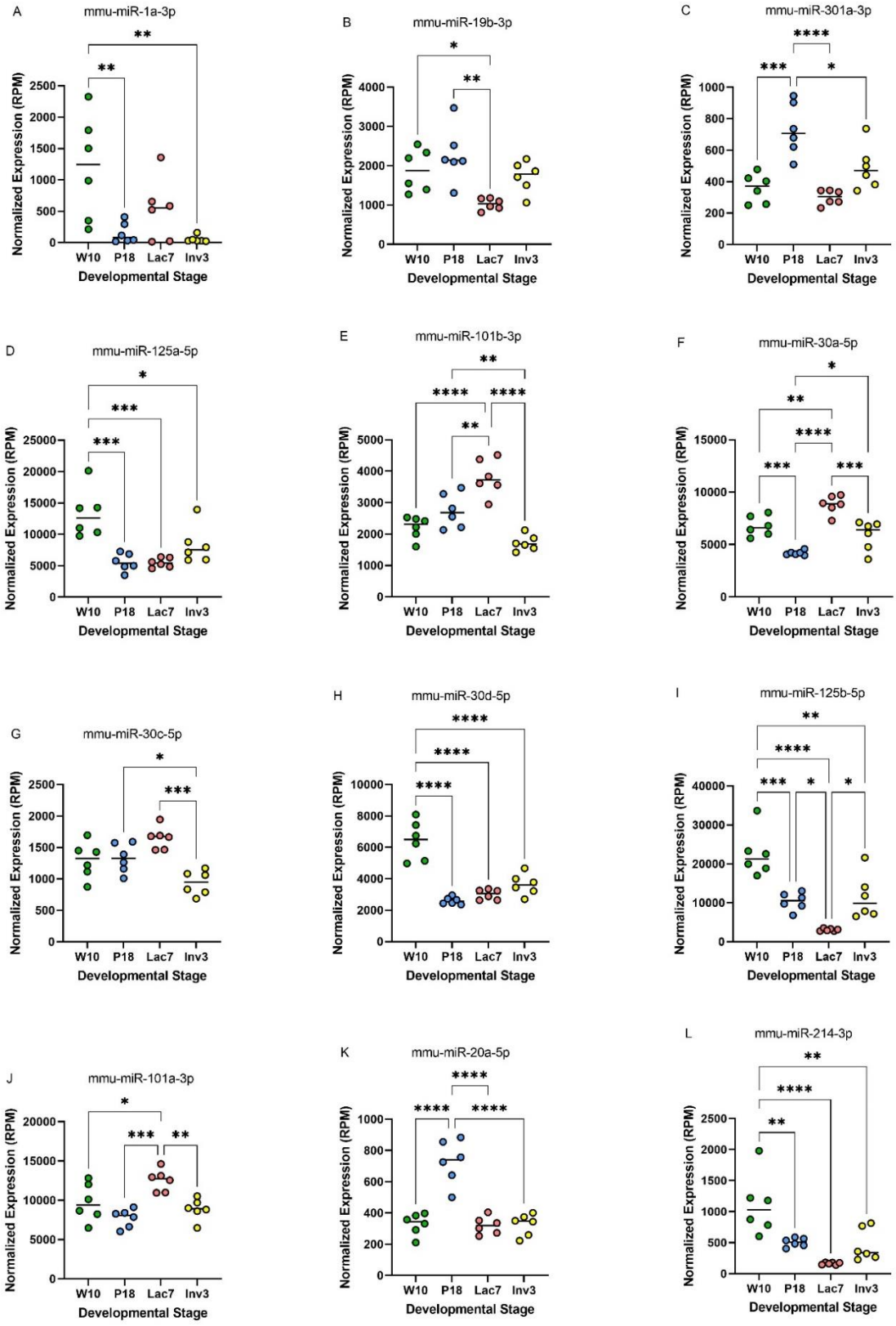
miRNA	Total Context Score
miR-1224-5p	-0.38
miR-224-5p	-0.37
miR-382-3p	-0.37
miR-582-5p	-0.29
miR-323-3p	-0.28
miR-325-3p	-0.25
miR-875-5p	-0.22
miR-329-3p/362-3p	-0.21
miR-28-3p	-0.21
miR-1197-3p	-0.21

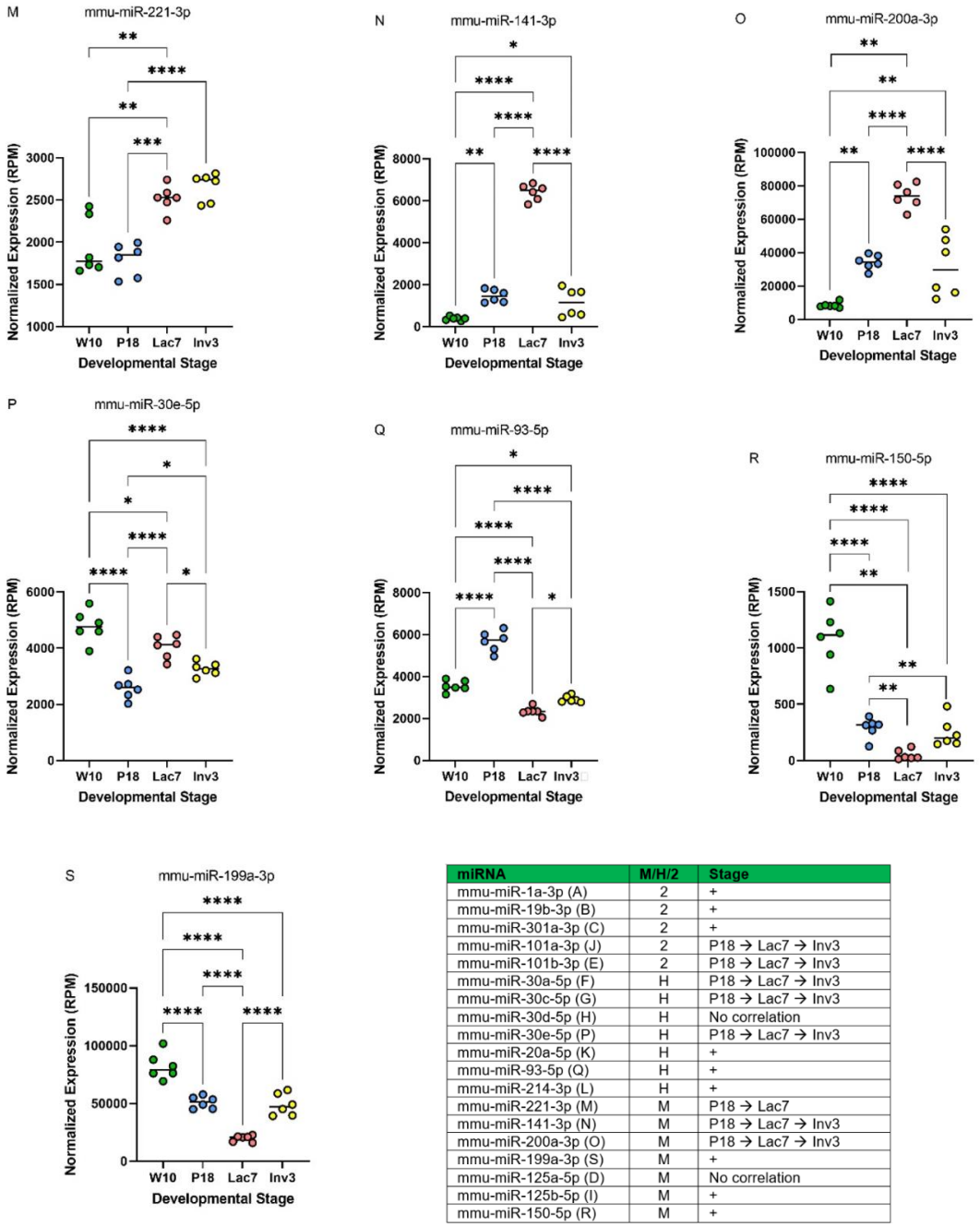
miRNA	Total Context Score
miR-877-3p	-0.44
miR-470-3p	-0.4
miR-883a-5p	-0.38
miR-3102-3p	-0.36
miR-3079-3p	-0.34
miR-466dk-5p	-0.33
miR-3078-5p	-0.3
miR-1930-5p	-0.29
miR-434-3p	-0.29
miR-3086-3p	-0.29
miR-674-3p	-0.28
miR-3084-3p	-0.28
miR-3077-3p	-0.27
miR-574-5p	-0.27
miR-3082-3p	-0.26
miR-672-5p	-0.25
miR-1188-5p	-0.25
miR-598-3p	-0.24
miR-574-3p	-0.23
miR-3094-5p	-0.21
miR-547-3p	-0.2

Figure 1: Potential miRNA regulating Cx43 mRNA based on the databases TargetScan Human 8.0 and TargetScan Mouse 8.0. Databases were searched for potential miRNA regulators of Cx43 (GJA1 gene) mRNA expression, and sorted based on total context score, using a maximum value of -0.20. Boxed miRNA are common to the human and mouse lists.

3.5.3 Cx43 mRNA and various miRNA regulator candidates are regulated by individual hormones in T-47D cells

We next wanted to understand how hormones can regulate miRNA and Cx43 expression. To do so, T-47D cells were treated with prolactin, estradiol, progesterone, and oxytocin from 0 to 8 hours. Efficiency of the treatments was confirmed using downstream targets (Supplementary figure 1). 38, 4, 24 and 66 miRNAs were significantly modulated by estradiol, progesterone,


A
mmu-miR-1a-3p
B
mmu-miR-19b-3p
C
mmu-miR-301a-3p
D
mmu-miR-125a-5p
E
mmu-miR-101b-3p
F
mmu-miR-30a-5p
G
mmu-miR-30c-5p
H
mmu-miR-30d-5p
I
mmu-miR-125b-5p
J
mmu-miR-101a-3p
K
mmu-miR-20a-5p
L
mmu-miR-214-3p
Normalized Expression (RPM)
W10
P18
Lac7
Inv3
Developmental Stage



miRNA	M/H/2	Stage
mmu-miR-1a-3p (A)	2	+
mmu-miR-19b-3p (B)	2	+
mmu-miR-301a-3p (C)	2	+
mmu-miR-101a-3p (J)	2	P18 → Lac7 → Inv3
mmu-miR-101b-3p (E)	2	P18 → Lac7 → Inv3
mmu-miR-30a-5p (F)	H	P18 → Lac7 → Inv3
mmu-miR-30c-5p (G)	H	P18 → Lac7 → Inv3
mmu-miR-30d-5p (H)	H	No correlation
mmu-miR-30e-5p (P)	H	P18 → Lac7 → Inv3
mmu-miR-20a-5p (K)	H	+
mmu-miR-93-5p (Q)	H	+
mmu-miR-214-3p (L)	H	+
mmu-miR-221-3p (M)	M	P18 → Lac7
mmu-miR-141-3p (N)	M	P18 → Lac7 → Inv3
mmu-miR-200a-3p (O)	M	P18 → Lac7 → Inv3
mmu-miR-199a-3p (S)	M	+
mmu-miR-125a-5p (D)	M	No correlation
mmu-miR-125b-5p (I)	M	+
mmu-miR-150-5p (R)	M	+

Figure 2: *In vivo* expression of bioinformatics candidates for Cx43 regulation. miRNA were extracted from mouse mammary glands at week 10 (W10), pregnancy day 18 (P18), lactation day 7 (Lac7) and involution day 3 (Inv3), and sequenced (n = 6). Candidates for Cx43 regulation were then selected based on a) Identification as a potential Cx43 regulator via the bioinformatics analysis, b) Levels above 500 reads per million (RPM). Graphs represent the levels in each animal per group; an ANOVA was done to evaluate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$). In addition, the bottom table shows correlations between miRNA candidates and Cx43 mRNA, stage of correlation and whether the candidate was from mouse (M), human (H) or both (2) databases. '+' indicates a strong positive correlation between a given miRNA and Cx43 mRNA.

prolactin, and oxytocin, respectively (Confuorti et al., submitted). Within this list, 3 potential miRNA involved in Cx43 regulation were modulated by estradiol: hsa-miR-125b-5p and hsa-miR-30c-5p were down-regulated, while hsa-miR-101-3p was up-regulated (Figure 3A). 4 miRNA were down-regulated upon prolactin exposure: hsa-miR-30c-5p, hsa-miR-125b-5p, hsa-miR-200a-3p and hsa-miR-93-5p (Figure 3C). 4 miRNA were up-regulated by oxytocin (has-miR-200a-3p, has-miR-101-3p, has-miR-141-3p, has-miR-30e-5p), and has-miR-125a-5p was up-regulated (Figure 3D). None of the candidates from Figure 1 and 2 were characterized as linked with progesterone exposure.

The expression of Cx43 was then assessed by RT-qPCR. Prolactin increased Cx43 mRNA expression after 2 hours of exposure; however, by the time point of 8 hours, Cx43 was decreased compared with the control (Figure 4A). Estradiol increased the expression of Cx43 in a dose-dependent manner, nearly reaching significance at 8 hours ($p = 0.08$) (Figure 4B). Similarly, progesterone significantly increased Cx43 expression starting from 4 hours and reaching significance at 8 hours (Figure 4C). Though oxytocin 100 nM treatment resulted in a sharp decrease in Cx43 expression after 2 hours (Figure 4D), this decrease was significant only at 2 and 6 hours. Thus, comparing Cx43 and miRNA expression upon hormonal exposure, prolactin decreased Cx43 expression and down-regulated 4 miRNA, estradiol increased Cx43 expression, down-regulated 2 miRNA and up-regulated 1 miRNA, and oxytocin decreased Cx43 expression while up-regulating 4 miRNA and down-regulating 1 miRNA.

3.6 Discussion

Results from our lab and from others have demonstrated that Cx43 plays a crucial role in mammary gland development and function, and is dysregulated in breast cancer. However, the mechanisms regulating Cx43 transcription in the mammary gland, including the role of miRNA, are poorly known. In this study, we aimed to unravel the links between miRNA, hormones, and Cx43 regulation. Hormone-linked miRNA that potentially regulate Cx43 mRNA expression were identified based on a) prediction of mRNA-miRNA interactions using online databases, b) inverse correlation between miRNA and Cx43 mRNA *in vivo,* c) change in expression of miRNA upon hormone exposure *in vitro,* and d) the effects of the same hormones on Cx43 mRNA expression *in vitro*. This led to identification of 9 inversely correlated and 7 positively correlated miRNA with Cx43 mRNA expression (Figures 1 and 2) (Dianati et al. 2016). In addition, 3, 0, 4 and 5 of these miRNA were linked with estradiol, progesterone, prolactin and oxytocin exposure, respectively (Figure 3). Cx43 mRNA expression was increased in response to estradiol, progesterone and 2

Estradiol (A)			Progesterone (B)		
miRNA	*LFC*	*p-value*	*miRNA*	*LFC*	*p-value*
hsa-miR-125b-5p	-0.34	0.001			
hsa-miR-30c-5p	-0.24	0.004			
hsa-miR-101-3p	0.30	0.004			
Prolactin (C)			**Oxytocin (D)**		
miRNA	*LFC*	*p-value*	*miRNA*	*LFC*	*p-value*
hsa-miR-30c-5p	-0.30	0.01	hsa-miR-200a-3p	0.41	0.0006
hsa-miR-125b-5p	-0.35	0.02	hsa-miR-101-3p	0.36	0.0009
hsa-miR-200a-3p	-0.23	0.03	hsa-miR-141-3p	0.70	0.001
hsa-miR-93-5p	-0.21	0.05	hsa-miR-30e-5p	0.23	0.03
			hsa-miR-125a-5p	-0.19	0.05

Figure 3: miRNA candidates for Cx43 regulation associated with estradiol, progesterone, prolactin and oxytocin exposure in breast cells. miRNA were extracted from T-47D cells exposed to (A) estradiol 10 nM, (B) progesterone 10 nM, (C) prolactin 8.69 nM (200 ng/mL) and (D) oxytocin 100 nM, and sequenced. Only miRNA differentially expressed upon hormonal treatment (LFC = log 2-fold change, from DESEQ2; p-value $\leq$0.05) and identified as potential candidates for Cx43 regulation, based on figures 1 and 2, are listed.

hours prolactin exposure, and decreased upon oxytocin exposure and 8 hours prolactin exposure (Figure 4). As miRNA expression is typically associated with decreased expression of mRNA targets, these results suggest that miR-125b-5p and miR-30c-5p down-regulation by estradiol can be associated with increased expression of Cx43. Similarly, these two miRNA could potentially have a short term effect on Cx43 mRNA upon exposure to prolactin, as its expression is decreased after 2h of exposure, but increased at 8h. Similarly, oxytocin can down-regulate miR-125b-5p expression, but up-regulate 4 other miRNA, resulting in an overall decrease in Cx43 expression. Thus, although these miRNA seem to be important for Cx43 regulation, more studies are needed to understand the complex interplay linking hormones and miRNA to Cx43 during mammary gland development.

3.6.1 Bioinformatics-predicted candidates for Cx43 regulation differ in expression between various stages of mouse mammary gland development

We first used the databases TargetScan Human 8.0 and TargetScan Mouse 8.0 to determine potential miRNA-mRNA interactions. Within this list of candidates, 19 miRNA were expressed above 500 RPM *in vivo*, which is considered functionally relevant (Mullokandov et al. 2012). In addition, all 19 miRNA were differentially expressed, including some that had already been

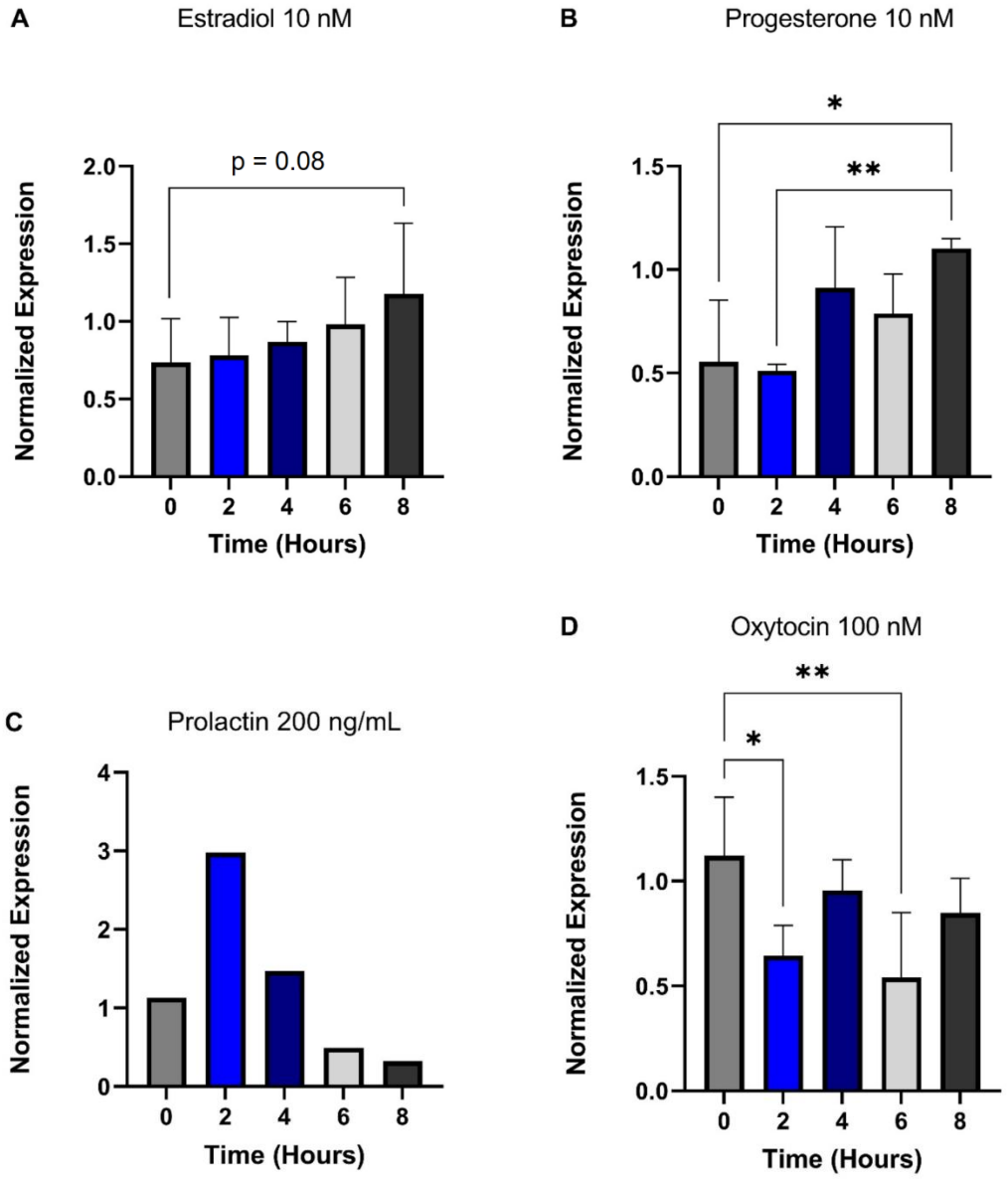


Figure 4: Effect of hormones on Cx43 mRNA expression in breast cells. Based on previous literature and experiments, T-47D cells were treated with estradiol 10 nM, progesterone 10 nM, prolactin 200 ng/mL and oxytocin 100 nM individually for 0 to 8 hours. Then, Cx43 mRNA was quantified by RT-qPCR. CFX software (Bio-Rad) was used to calculate Cx43 mRNA expression, using the $\Delta\Delta C_t$ method ($n \geq 4$). For PRL, preliminary data are shown ($n = 1$). Stable reference genes used in the normalization included UXT, RPL13A and HPRT1.

identified in other studies, supporting a potential stage-specific role in Cx43 regulation. Of those 19 differentially expressed miRNA, 8 were inversely correlated and 9 were positively correlated with Cx43 mRNA expression at lactation (Dianati et al. 2016).

Cx43 was already identified as a potential target for some of those miRNA in other studies. For example, miR-200a-3p was determined a strong predicted target of Cx43 regulation in TargetScan Mouse 8.0, but not TargetScan Human 8.0. miR-141-3p and miR-200a-3p both sharply peaked during lactation, where Cx43 expression is lowest (Dianati et al. 2016). It has been suggested that the miR-200 family regulates Cx43, which could contribute to lactation (Roth and Moorehead 2021). This is concurrent with other literature showing elevated expression of this miRNA family at this stage (Li et al. 2012). Ming et al. 2015 also showed that miR-200a-3p downregulates Cx43 directly via the 3' UTR of its mRNA in breast cancer cells. Thus, literature suggests that miR-200a-3p could be directly implicated in mammary gland function at lactation by silencing Cx43. In addition, miR-200a-3p was up-regulated upon oxytocin exposure in human T-47D cells in our study, further supporting a role in regulating Cx43 in human breast.

As another example, miR-30c-5p peaked at lactation and was thus negatively correlated with Cx43 mRNA at that stage (Dianati et al. 2016). A negative correlation was also observed between miR-30c-5p and Cx43 in rat aortic endothelial cells, likely resulting from the direct binding of miR-30c-5p to Cx43 mRNA via its 3' UTR as demonstrated by dual-luciferase reporter assays, further supporting involvement of this miRNA in Cx43 expression during lactation (Yang et al. 2023).

miR-125b-5p was a predicted candidate for Cx43 regulation based on bioinformatics analyses and gene enrichment analyses in a study by Suzuki et al. 2022. Evidence shows that this miRNA is also affected by AMPK in HEK293 cells, which can increase its transcription, regulating Cx43 mRNA (Dufeys et al. 2021). Concurrent with the complexity of Cxs' regulation, miR-125b-5p has positive or negative effects on Cx43 expression depending on the cell line tested (Jin et al. 2013). In our study, miR-125b-5p was negatively correlated with Cx43 expression upon estradiol treatment and 2h after prolactin treatment, but positively correlated with Cx43 expression upon oxytocin treatment and 8h after prolactin treatment, further supporting a complex role of this miRNA in Cx43 regulation.

To the best of our knowledge, the other miRNA that were identified both *in silico* and from our sequencing study have not yet been identified yet in other studies. The direct link between Cx43 regulation and their expression thus need additional studies to be validated.

3.6.2 Some candidates for Cx43 regulation are affected by hormones in T-47D cells

Similarly, some miRNA that were identified as regulated by hormones in our study have already been linked with hormones in other studies, either as regulator or targets of hormone signaling.

For example, it has been demonstrated that the miR-200 family directly targets ZEB1 via its 3' UTR, causing its downregulation (Bracken et al. 2008, TargetScan Human 8.0). In the uterus, ZEB1 expression increase resulted in increased ZEB2 (Renthal et al. 2010), and both proteins suppressed Cx43, oxytocin receptor (OXTR) and the miR-200ab/429 family via direct recruitment of co-repressors to their promoters (Renthal et al. 2010). This occurred concurrent with an upregulation of progesterone at gestation, whereas decreased progesterone resulted in decreased ZEB1/2 and increased miR-200 and contractile genes at labour (Renthal et al. 2010). In our study, oxytocin treatment resulted in increased expression of miR-200a-3p, thus suggesting that miR-200a-3p might be involved in milk ejection and be linked with oxytocin and Cx43 at this stage, not just in the uterus, but also the mammary gland. Interestingly, miR-141-3p peaked in expression at the same time as miR-200a-3p, and was also increased by oxytocin. This could be expected, as miR-141-3p is part of the same family as miR-200a-3p (Cavallari et al. 2021), further supporting a role of these miRNA in lactation and regulation by hormones at this stage. In human myometrial tissue samples of women administered oxytocin to induce labor, expression of 12 miRNA, including hsa-miR-141-3p, were altered compared with tissue samples analyzed prior to labour (Cook et al. 2015).

In the current study, we observed decreased hsa-miR-30c-5p upon estradiol exposure. Exposure to estradiol from 6 to 72 hours resulted in decreased miR-30c in both MCF-7 and ZR-75.1 cells (Ferraro et al. 2012), as well as in uterine cell lines (Kong et al. 2014). In endometrial cancer, miR-30c is frequently dysregulated; authors of one study linked estradiol exposure with downregulation of this miRNA, in Ishikawa and HEC-1-B cells (Kong et al. 2014). Further, hsa-miR-30c-5p was downregulated in MCF-12A (estradiol-non-responsive) vs. MCF7 (estradiol-responsive) cells (Bailey, Westerling and Brown 2015). Of note, profiling studies identify links between estradiol and other miR-30 family members than just hsa-miR-30c-5p, further supporting a potential link between this miRNA and hormones (Liu et al. 2019; Bailey, Westerling and Brown 2015).

In MCF7 cells, a miR-125b downregulation began at 6 hours upon estradiol exposure and was more pronounced at 72 hours (Ferraro et al., 2012). However, in a different study using MCF7 cells, estradiol had the opposite effect, possibly due to different treatment time used (4 hours) (Bhat-Nakshatri et al. 2009). We also observed an up-regulation of miR-125b-5p upon estradiol treatment. It has been demonstrated that estradiol-induced increase of miR-125b resulted in downregulation of NF-κB in macrophages stimulated with lipopolysaccharide (Murphy, Guyre and Pioli 2010). hsa-miR-125b-5p was upregulated in MCF712A (E_2-non-responsive) vs. MCF7 cells

(E_2-responsive) (Bailey, Westerling and Brown 2015). However, in splenic white blood cells, estradiol displays the opposite effect on miR-125b (Dai et al. 2008), demonstrating the complexity of miRNA regulation by hormones in different cells.

miR-101-3p expression was increased in our study upon estradiol exposure, similar to what has been observed before in MCF-7 cells (Castellano et al., 2009). Interestingly, this effect was observed after 6 hours of treatment, but not 12 hours, suggesting a transient effect (Castellano et al. 2009). In a different study, miR-101-3p was unaffected after 24 hours by treatment (Ferraro et al. 2012), confirming that the effect of estradiol on miR-101-3p is time-dependent.

For prolactin exposure and miRNA, not many studies exist, but information can still be gathered. In one of the articles, 4 miRNA were differentially expressed upon exposure; however, the authors used RT-qPCR, which limit the number of potential candidates compared with sequencing (Lin et al. 2013). Another study using RT-qPCR methods found 7 altered miRNA in either mammary epithelial cells or their media upon combined treatment with dexamethasone, insulin and prolactin (Muroya et al. 2016). In another, 42 miRNA were identified as differentially expressed upon prolactin exposure, using miRNA sequencing analyses (Wei et al. 2013). When comparing identified miRNA amongst the studies and the current project, miRNA were scarcely common to the lists, but some family members appear more than once, such as miR-200 (Lin et al. 2013) and miR-23 (Lin et al. 2013; Muroya et al. 2016), supporting a potential role of these miRNA in gene expression changes in response to prolactin.

3.6.3 Cx43 mRNA expression is tied with miRNA and hormone action in T-47D cells

This study found a tendency of positive association between exposure to estradiol and Cx43 expression. This is concurrent with other studies showing positive association between estradiol (Ren et al. 2013) and its receptor (Tsai et al. 2018) with Cx43. GJA1 also contains predicted response elements of estradiol in its promoter, which were confirmed with a luciferase assay (Yu, Dahl and Werner, 1994). At the same time, many miRNA were also activated/deactivated by estradiol. In particular, hsa-miR-125b-5p and hsa-miR-30c-5p were decreased by this hormone, and were candidates for Cx43 regulation. hsa-miR-101-3p was increased by the exposure (Figure 3A), and was also a candidate for Cx43 regulation. Decreased expression of hsa-miR-30c-5p in response to estradiol and inverse correlation of mmu-miR-30c-5p to Cx43 mRNA suggests that estradiol induction of Cx43 mRNA may be through suppression of miR-30c-5p transcription. Prior to lactation, estradiol remains elevated and miR-30c-5p low, which may be keeping Cx43

expression high (Dianati et al. 2016). At lactation, estradiol decreases and miR-30c-5p increases, which appears to decrease Cx43 expression (Dianati et al. 2016).

Our data suggest that GJA1 is positively regulated by progesterone. In the myometrium, some studies show negative association between Cx43 and progesterone (Petrocelli and Lye 1993), including one study that demonstrated down-regulation of GJA1 transcription via interaction of progesterone receptor (PR) with AP1 (Dong et al. 2009, Xie et al. 2012). A recent study elaborated that PRα and PRβ have differing effects on Cx43 expression, but can even enable the expression of 20 KDa Cx43, a truncated form linked with full-length Cx43 localization (Nadeem et al. 2017).

Prolactin decreased Cx43 mRNA expression after 8 hours of exposure. In addition, of 4 candidates for Cx43 regulation associated with prolactin exposure, 4 miRNA (hsa-miR-30c-5p, hsa-miR-125b-5p, hsa-miR-200a-3p and hsa-miR-93-5p) were downregulated. Decreased miRNA expression through prolactin should cause an increase in Cx43, which is not seen at lactation (Dianati et al. 2016); thus, it cannot be confirmed whether or not Cx43 regulation by miRNA is linked with circulating prolactin. The above miRNA could be linked with 2 hours prolactin exposure, where Cx43 mRNA increases, but more studies will be needed to confirm this. Nonetheless, Cx43 and miRNA could be linked with milk component synthesis, largely regulated by prolactin (Macias and Hinck 2012). Authors of a recent study carried out a Cx43 KO targeted to adipocytes that resulted in reduced lactose content in mammary gland milk, leading to smaller offspring in mice (Huang et al. 2022). However, the mechanism by which lactose decreased in milk was not conclusive, so it may be due to another factor than nutrient exchange through GJs. GJs are permeable to milk components such as Ca^{2+}, Mg^{2+}, water, miRNA and siRNA (Sàez et al. 1989; Dror and Allen 2018; Martin, Ling and Blackburn 2016; Zong et al. 2016 and Wang et al. 2016). Further, treatment done in mammary epithelial cells with cAMP resulted in induced transcription of β-casein in a STAT5-independent mechanism (Talhouk et al. 2011). Thus GJIC and milk-component synthesis could be linked, as cAMP is a transcription factor that induces Cx43, but more studies on this topic are needed (Oyamada M., Takebe and Oyamada Y. 2013). Overall, there appear to be some links between prolactin, Cx43, miRNA and milk component synthesis, but further studies are needed to confirm them.

In this study, oxytocin resulted in decreased Cx43 mRNA after 2 and 6 hours of exposure of breast cells (Figure 4D). Oxytocin induces contraction of myoepithelial cells during lactation, permitting the ejection of milk from inner luminal cells (Plante, Stewart and Laird 2011; Haaksma, Schwartz and Tomasek 2011). However, contraction is short-lived (Raymond et al. 2011), because feeding and the suckling stimulus do not last long, and the gland contains limited milk. Thus, it is expected

that pathways exist to rapidly deactivate oxytocin effects, which could result in short-term Cx43 mRNA changes. One possible mechanism is receptor recycling, whereby rapid re-expression of a receptor occurs through sorting and/or recycling endosomes near the membrane (Goldenring et al. 2015; O'Sullivan and Lindsay 2020). For example, Conti et al. 2009 showed that oxytocin receptors stably expressed in HEK293T cells were quickly internalized after agonist exposure, but re-visible again in the membrane after 4 hours, when the cell regained sensitivity to the agonist. Receptors were not co-localized with the lysosome, suggesting that endosome recycling, and not degradation/receptor biogenesis was responsible for the re-expression. In fact, the short-lived nature of contraction makes miRNA plausible effector molecules of oxytocin-mediated contraction involving Cx43, because like Cx43, they have a rapid biogenesis and half-life (Reichholf et al. 2019). Of 5 candidates for Cx43 regulation linked with oxytocin exposure (Figure 3D), 4 (hsa-miR-200a-3p, hsa-miR-101-3p, hsa-miR-141-3p and hsa-miR-30e-5p) were upregulated, and 1 (hsa-miR-125a-5p) was downregulated. Increase of these 4 miRNA in response to oxytocin (Figure 3D) and inverse correlation between those four candidates and Cx43 expression (Figure 2O, J, E, N, P) (Dianati et al. 2016) suggests that Cx43 mRNA decrease via oxytocin may be effectuated through those miRNA. In fact, the oxytocin pathway is most active at lactation, where Cx43 expression is minimal (Dianati et al. 2016) and all 4 candidates are at peak expression. Normal level of Cx43 is needed for milk ejection (Plante and Laird 2008, Mroue et al. 2015), and oxytocin link with GJs has been demonstrated (Chan, Berezin and Daniel 1988). Thus, our data that show regulation of Cx43 by oxytocin is in agreement with the general consensus on their involvement in lactation and mammary gland function.

3.7 Limitations, perspectives and conclusion

A few limitations should be considered when analyzing the data. Firstly, breast cancer cells, rather than normal mammary gland cells, were used in the hormone analyses, to allow comparison of breast cancer to regular mammary glands. Though T-47D cells are less aggressive than other types of breast cancer cells (Chen, Ritsma and Vrisekoop 2019), the cancer status could affect response to hormones. In addition, T-47D cells are an effective model due to their expression of estradiol, progesterone, oxytocin and prolactin receptors (ATCC website, Fay et al. 1999) and Cx43 (Gakhar, Hua and Nguyen 2010), but are nonetheless only one cell type. In normal mammary glands, luminal cells exchange information with myoepithelial cells and the extracellular matrix, and are supported by growth signals from the stroma (Macias and Hinck 2012). In addition, limited amount of miRNA were identified as linked with progesterone exposure. This could be due

to different treatment time, concentration or cell line used, but most likely different progestin molecules (PubChem 2023) (medroxyprogesterone vs. progesterone) in the exposure (Cochrane et al. 2012). However, another factor is that progesterone is a highly synergistic hormone, especially with regard to prolactin and alveogenesis at lactation, and might thus require presence of other hormones for full miRNA regulation (Macias and Hinck 2012). Nonetheless, the identified miRNA for progesterone exposure point towards miRNA potentially regulated by progesterone in the mammary gland. Furthermore, since hormones regulate Cx43, and hormones can show short-term fluctuations, it could be that its expression may be different at stages of lactation different than those tested (Dianati et al. 2016). Thus, although other miRNA likely regulate Cx43, detection is restricted by the number of stages analyzed. Lastly, miRNA can display synergy amongst each other (Chen et al. 2017; Lin et al. 2013), meaning that groups of miRNA could participate in Cx43 regulation. Here, the link between group miRNA expression and hormones or Cx43 may not be detected, because our assessment analyzes one miRNA at a time.

In conclusion, this study found that several miRNA predicted to regulate Cx43 mRNA in online databases were inversely correlated to Cx43 mRNA expression. Cx43 mRNA was differentially regulated by the circulating hormones estradiol, progesterone, prolactin and oxytocin. In addition, some candidates for Cx43 regulation were linked with hormone exposure, suggesting that hormones could induce/suppress miRNA expression, affecting Cx43 and GJIC. Regulation of Cx43 by miRNA appears especially linked with oxytocin at lactation, where it may rapidly induce changes in GJIC, accommodating contractile properties of the mammary gland.

3.8 Acknowledgements

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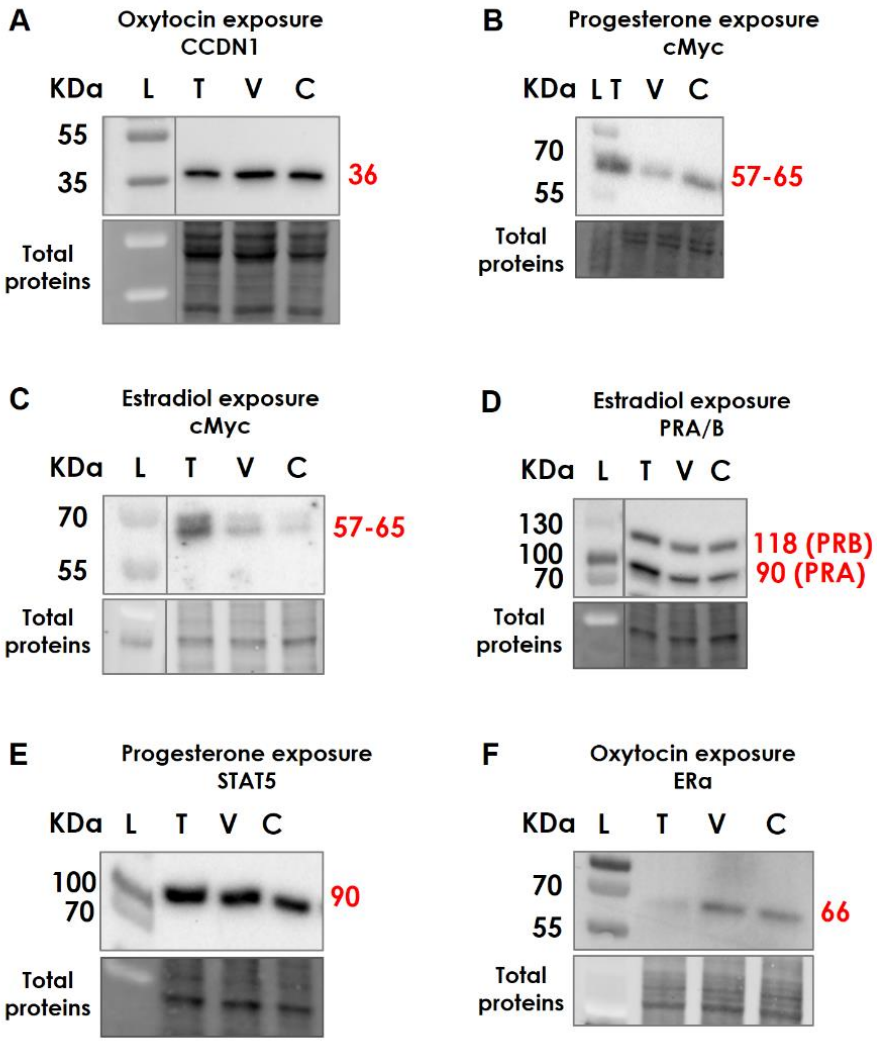
3.9 Supplementary material

Supplementary Table 1: Primers used in Cx43 mRNA quantification (RT-qPCR)

Gene	5’ to 3’ primer	3’ to 5’ primer	Ref(Y/N)
GJA1	TACCAAACAGCAGCGGAGTT	TGGGCACCACTCTTTTGCTT	N
UXT	TGAGCGACTCCAGGAAGCTA	GGGACCACTGTGTCAACGAA	Y
RPL13A	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA	Y



Supplementary figure 1: Effect of hormones on select markers in T-47D cells. Based on previous literature and experiments, T-47D cells were exposed to estradiol, progesterone and oxytocin. Downstream markers were evaluated by Western blot: for estradiol exposure, cMyc and PRA (expected upregulation) (Rangel, Villegas and Rondon-Lagos 2017), for progesterone exposure, cMyc and STAT5 (expected upregulation) (Kougioumtzi, Tsaparas and Magklara 2014), and for oxytocin exposure, ERα and CCND1 (expected downregulation) (Cassoni et al., 2002; Khori et al. 2018). One replicate of three (n = 3) is shown; if showing the second or third replicate, the blot was cropped and ladder moved next to that replicate, without changing the vertical position. T = treatment (hormone), V = vehicle, C = untreated control.

Supplementary Table 2: Antibodies used in T-47D markers quantification (Western blot)

Antibody	Company	Catalogue #	Species	Dilution	Blocking
c-Myc	Cell Signaling	5605	Rabbit	1:2000	5% milk powder
Progesterone receptor	Cell Signaling	8757	Rabbit	1:1000	5% milk powder
Estrogen receptor α	Cell Signaling	8644	Rabbit	1:1000	5% BSA
Cyclin D1	Cell Signaling	2922	Rabbit	1:1000	5% milk powder
STAT5	Cell Signaling	94205	Rabbit	1:1000	5% milk powder
Anti-rabbit IgG-HRP	Cell Signaling	7074	Goat	1:10000	5% milk powder

3.10 References

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CHAPTER 4 : GENERAL DISCUSSION

Our lab and other researchers' findings have demonstrated that Cx43 is indispensable to the formation and function of the mammary gland. Nevertheless, little is known about the factors controlling its expression, including the role of miRNA. In addition, Cx43 and miRNA are both implicated in breast tumour progression and metastasis. The goal of this work was to elucidate the relationships among hormones, miRNA and Cx43 regulation. Using bioinformatics approaches, we were able to classify expressed miRNAs into six clusters, shedding light on broad fluctuations of their levels across development. In addition, individual miRNAs vary greatly between stages, especially at lactation, visible in their expression levels and miRNA that were uniquely found at one stage. As anticipated, many processes linked with remodeling and hormones were associated with differentially expressed miRNA. E_2, P_4, PRL and OXT were found to be linked with miRNA; usually, the miRNA linked with a particular hormone was not regulated by others, but regulation of the same miRNA by more than one hormone was found in some instances. With these results, we identified 16 miRNA potentially regulated by hormones in a stage-specific manner.

miRNA linked with hormones that potentially regulate Cx43 were identified based on the following: a) prediction of mRNA-miRNA interactions using online databases; b) inverse correlation between miRNA and Cx43 mRNA *in vivo*; c) change in miRNA expression upon hormone exposure *in vitro*; and d) effects of the same hormones on Cx43 mRNA expression *in vitro*. Nine of the identified miRNA in databases were inversely correlated to Cx43 mRNA levels based on a previous study from our lab. Some of those miRNA were also linked with the exposure to hormones, especially oxytocin at lactation. In addition, Cx43 mRNA was increased in response to estradiol and progesterone after 8 h exposure, and decreased by oxytocin after 2 and 6 h exposure. These results led to identification of hormonally associated miRNA affecting Cx43 expression, which will be further analyzed in future studies.

4.1 Broad expression patterns of miRNA suggest wide-ranging functions in mammary gland development

Data on differential expression of miRNA *in vivo* suggests that like proteins, mammary gland miRNA are ubiquitous as a group in the tissue, but as individual species they are uniquely expressed, and also play important, wide-ranging functions.

4.1.1 Bioinformatics analyses are useful in identifying potential candidates for Cx43 regulation

The databases TargetScan Human 8.0 and TargetScan Mouse 8.0 determine potential miRNA-mRNA interactions. Several miRNA predicted to target Cx43 mRNA have been validated *in vivo*, often using miRNA inhibition and/or overexpression experiments, and *in vitro*, often using inhibition or overexpression experiments and luciferase assays. For instance, mice engineered to express a tetracycline-inducible overexpression vector for miR-130a showed a 90% reduction of Cx43 after 10 weeks, which resulted in tachycardia and atrial and ventricular arrhythmias (Osbourne et al. 2014). In the same study, Cx43 was directly targeted via the 3' UTR of its mRNA by miR-130a, tested using a luciferase assay in 3T3 and HL1 cells. miR-221/222 targeted Cx43 mRNA directly via its 3' UTR, and liposome transfection of a miR-221/222 inhibitor decreased invasion and proliferation and increased apoptosis in glioblastoma U251 cells (Hao et al. 2012). Thus, miR-221/222 partly contributes to glioma properties via regulation of Cx43 (Hao et al. 2012). miR-206 and miR-200a-3p also directly interacted with Cx43 mRNA at the 3' UTR, regulating its expression (Jin et al. 2019; Ming et al. 2015a). However, despite these studies, evidence for Cx43 regulation by miRNA in the mammary gland was nonetheless scarce. Thus, as a first step, we set out to determine which bioinformatics candidates for Cx43 regulation could be inversely correlated with Cx43 mRNA *in vivo* (Dianati et al. 2016).

4.1.2 Broad expression patterns of miRNA

Bioinformatics analyses revealed six developmental clusters representing general patterns of miRNA expression across W10, P18, Lac7 and Inv3. Clusters with the most miRNA reached maximum expression (Cluster 1, 93 genes) or minimum expression (Cluster 3, 135 genes) at Lac7, suggesting that these groups of miRNA could be involved in lactation regulation through their silencing or increased expression of mRNA transcripts. Some of those miRNA drastically changed in expression at Lac7, which hints that miRNA might not only involved in fine-tuning of homeostasis, but also in mammary gland function, as lactation is the stage where milk delivery occurs (Do et al. 2017). However, groups linked with stages other than lactation and with more varied patterns of expression were also present, which suggests universal use of miRNA in cells as a gene expression regulation mechanism. For example, Cluster 5 shows minimum miRNA expression at W10 and maximum miRNA expression at P18, suggesting importance of this group at pregnancy, rather than lactation. Cluster 6 is more unique, beginning at maximum expression

at W10, then decreasing to moderate expression at P18 and Lac7, and minimal expression at Inv3, suggesting potential importance of this group at W10 or Inv3. miRNA stage-specific functions are also highlighted by miRNA exclusively detected at one stage, which were identified for W10 (33 miRNA), P18 (15 miRNA), Lac7 (seven miRNA) and Inv3 (three miRNA).

4.1.3 Comparison of broad miRNA expression patterns with literature

The expression patterns of miRNA in dairy goat mammary glands has been analyzed at stages of late gestation, late lactation and dry period (Xuan et al. 2020). Similarly to our results, the dataset of the authors was categorized into six expression clusters, where miRNA expression at late lactation largely differed from dry period and late gestation, which supports miRNA involvement in lactation suggested by our data. This same idea is further supported by Li et al. 2012b, who found 56 differentially expressed miRNA in lactating vs. non-lactating bovine mammary glands. In summary, miRNA groups showing minimal or maximal expression at lactation or other stages were identified using bioinformatics analyses, unveiling the potential wide-ranging importance of miRNA not just in mammary gland function (ie. breast milk delivery), but also in growth and remodeling.

4.2 Individual expression patterns of miRNA propose that some miRNA as individuals are required for development and lactation

Broad expression patterns of miRNA implicate them as central players in the central dogma of molecular biology. Examining more closely the expression patterns of individual miRNA *in vivo*, some miRNA are likely indispensable as individuals or families to mammary gland development. miRNA that play only minor functions may not be required for mammary gland development, but collective loss of those miRNA could still result in deleterious mammary gland development.

4.2.1 miR-200 family

In our study, the five members of the miR-200 family (Humphries et al. 2015) displayed peak expression at lactation. mmu-miR-200a-3p, 200b-3p, 141-3p and 429-3p increased in expression from P18 to Lac7 by 2, 2, 4 and 3-fold, respectively. In addition, those four miRNA were among the group of top 30 differentially expressed genes from both P18 to Lac7 and Lac7 to Inv3. In Xuan et al. 2020, differential expression of chi-miR-200b-3p and chi-miR-200c-3p in dry period compared with the other two stages was identified, and Li et al. 2012b identified miR-

200a-3p expression as maximal at lactation compared with other stages. Our study thus supports high expression of the miR-200 family at lactation shown in literature and suggests its possible requirement for lactation in the mammary gland. In normal mammary gland stem cells found in close proximity to tumours, miR-200c was associated with reduced ability of cells to form ducts (Shimono et al. 2009). In EpH4 mammary epithelial cells, miR-200a knockdown resulted in reduced casein beta and cadherin 1 expression upon exposure to lactogenic hormones, relative to control treatments, and increased ZEB1 (Nagaoka et al. 2013). Using a 3D cell culture model, it was also found that processes analogous to formation of ducts were negatively affected by miR-200 knockdown, as well as claudin 3 and par-6b, markers of epithelial cell polarity. In particular, miR-200a was found in one study to target 917 transcripts in MDA-MB-231 breast cancer cells, suggesting a broader importance than just gene expression regulation at lactation (Bracken et al. 2014). Among those targets are the ZEB and Snai families of transcription factors as well as Twist, involved in epithelial-mesenchymal transition properties of cells (Park et al. 2008; Korpal et al. 2008). Epithelial cell polarity and epithelial-mesenchymal properties affect cell proliferation within the surrounding fat pad early in development (May et al. 2011). β-catenin, an interactor of Cx43 within a structure termed the junctional nexus, which links gap, adherens and tight junctions at the plasma membrane (Dianiti et al. 2016), is also a direct target of miR-200a (Saydam et al. 2009; Su et al. 2012). Collectively, our study and other literature strongly suggest potential requirement of the miR-200 family not just for lactation, but mammary morphogenesis and remodeling, namely duct elongation. Further studies are needed to determine its effect on lactation.

4.2.2 miR-30 family

Our study supports the notion that miR-30 is associated with lactation. miR-30 is predicted to target Cx43 mRNA *in silico*, and was also differentially expressed throughout the four stages of mammary gland development. For example, mmu-miR-30c-5p differed in expression from P18 to Lac7, and Lac7 to Inv3, showing peak expression at Lac7 where luminal Cx43 expression is minimal (Dianiti et al. 2016). In a previous study, miR-30b overexpression resulted in smaller alveoli and lipid droplets at lactation, with many genes' expression altered (Le Guillou et al. 2012). Some of those defects persisted into involution, and offspring fed by the mothers showed reduced body weight, supporting a potential important role of this miRNA in lactation.

4.2.3 miR-101-3p

miR-101-3p suppresses proliferation and is under expressed in some breast cancer subtypes (Wang et al. 2012; Tanaka et al. 2008). In one study, miR-101a overexpression was associated with an increase in cyclooxygenase-2 expression and decrease in HC11 cell proliferation, but also a decrease in β-casein mRNA (Tanaka et al. 2008). In our data, miR-101-3p was highly expressed at Lac7 compared with all other stages. Based on this information, if miR-101-3p is involved in lactation, it is likely during milk ejection and not production, because β-casein (decreased by miR-101a) is needed as a component. miR-101-3p was also a top gene altered by OXT exposure, further suggesting a role during milk ejection.

4.2.4 miR-206

Previous works have identified miR-206 as a Cx43 regulator in heart, bone, muscle, ovary and breast cells (Jin et al. 2019; Zhang et al. 2020; Inose et al. 2009; Yu et al. 2020; Fu et al. 2015). Our *in vivo* study did not detect miR-206 at levels above background. This might be expected, based on two lines of evidence: a) literature suggests that this miRNA contributes to embryogenesis as opposed to other stages, and b) previous authors who sequenced mouse mammary glands at post-natal to involution stages found that miR-206 was only highly expressed pre-puberty and late involution (Avril-Sassen et al. 2009), both of which our study did not test. It is possible that this miRNA is required for mammary gland development, but expression must be confirmed with additional studies at the aforementioned stages.

4.3 Cx43 regulation by miRNA is a dynamic, multidimensional process

4.3.1 General model of regulation of Cx43 mRNA by miRNA

In vivo data show miRNA expression can be maintained as transcriptionally stable, playing homeostatic roles (Wagschal et al. 2015), or change rapidly to induce short-term effects on downstream genes (Reichholf et al. 2019). Cx43 regulation by miRNA is a dynamic process, affected by multiple factors and intricately linked with other cellular pathways (Figure 12). mRNA regulation by a given miRNA can be viewed as a standalone pathway, but in reality, such regulation is much more complex. When considering that individual miRNA can target multiple mRNA transcripts, such regulation is infinitely more complex.

4.3.2 Synergy of miRNA with the process of methylation and transcription factors

miRNA are themselves transcribed from genes in the nucleus, which means that regulatory pathways of each individual gene indirectly affects Cx43 expression. miRNA genes whose promoters contain CpG islands, regions rich in cytosine and guanine repeats, are affected by methylation status, affecting their level of transcription (Kwon et al. 2017; Dakhlallah et al. 2013; Lehmann et al. 2008; Lodygin et al. 2008). Thus, many unique miRNA affecting Cx43 or its interactome, could be differentially regulated by methylation. For example, miR-203a-3p, predicted to target histone deacetylase 1 mRNA *in silico* (TargetScan Human 8.0), has been shown to affect GJA1 promoter methylation (Zupkovitz et al. 2006), and is itself differentially expressed at involution (*in vivo* sequencing data). Thus, even though this miRNA does not target Cx43 mRNA, it can have an effect through methylation enzymes affecting Cx43.

In addition, miRNA genes are regulated by transcription factors (Gulyaeva et al. 2016), and miRNA and transcription factors regulate Cx43. For example, all five members of the miR-200 family have potential binding sites in the 3' UTR of JUN, a transcription factor of Cx43 (TargetScan Human 8.0). miR-200 is differentially expressed across stages, and some members are affected by estradiol, progesterone, prolactin or oxytocin (*in vivo* and *in vitro* sequencing data). Thus, the miR-200 family can affect Cx43 directly via the 3' UTR of its mRNA (Ming et al. 2015a), but also through transcription factors interacting with GJA1. Overall, differential expression of miRNA does not only affect Cx43 mRNA, but also has an impact on regulatory mechanisms that themselves regulate GJA1 (Figure 12).

4.3.3 Synergy of miRNA and the interactome of Cx43

An added layer of complexity is visible when considering the interactome of Cx43 (Figure 12). β-catenin, a factor that has been reported to directly induce GJA1 transcription (Xia et al. 2010), but also interacts with the protein (Dianati et al. 2016), can also be directly targeted by miR-200 (TargetScan Human 8.0 and Huang et al. 2010). E-cadherin and claudin-7, also interacting proteins of Cx43 (Dianati et al. 2016), could also be targeted by distinct sets of miRNA (TargetScan Human 8.0), which could ultimately affect Cx43 expression. β-catenin, E-cadherin and claudin-7 form part of the junctional nexus, the membrane structure formed by interactions between gap junctions, tight junctions and adherens junctions (Dianati et al. 2016).

4.3.4 Synergy of hormones with Cx43 and miRNA

In the hormone treatments and miRNA sequencing experiment, expression of distinct groups of miRNA were changed by E_2, P_4, PRL or OXT. *In vivo*, hormones themselves act in synergy, which is needed for full activation of their pathways. As for a role in mammary gland function, miRNA are plausible effector molecules of OXT-mediated contraction involving Cx43, because like Cx43, they have a rapid biogenesis and half-life (Reichholf et al. 2019), which could lead to flexibility in their action. Hormones carry out global regulation of genes (Figure 12), which could be involved in processes mentioned in the above sections, or Cx43 regulation itself (ie. negative regulation of Cx43 by oxytocin, or positive regulation by progesterone). Thus, overall, miRNA regulation of Cx43 and GJIC is a multidimensional and dynamic process. Conceptually, Cx43 regulation pathways can be visualized alone, but functionally, they are intricately tied together.

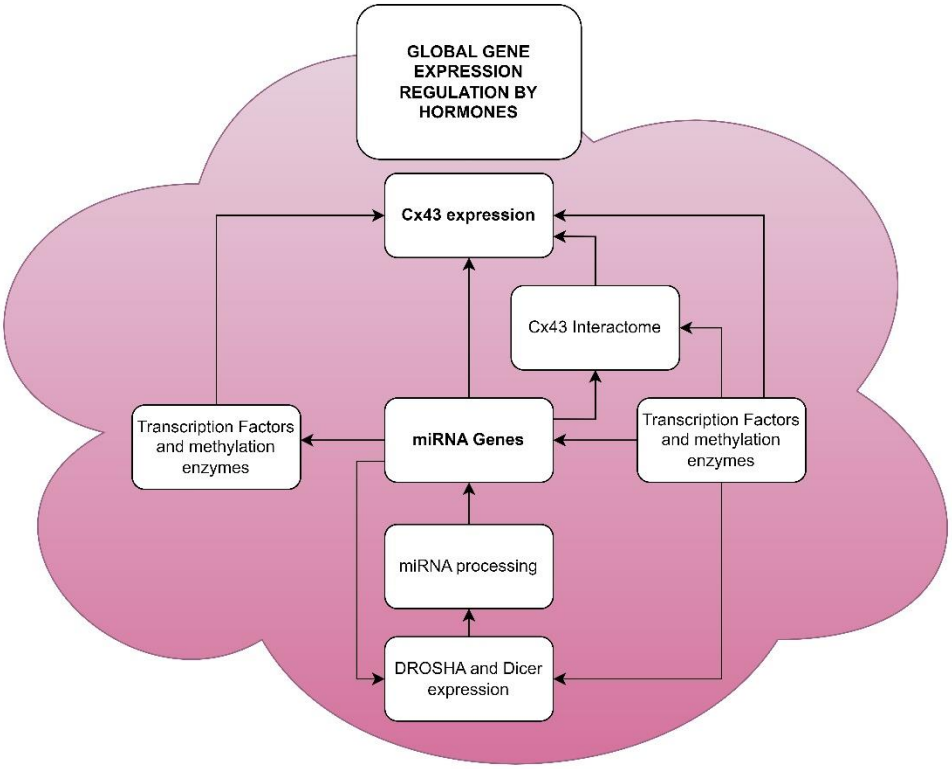

Figure 4.1: Complexity of Cx43 Regulation. Cx43 regulation by miRNA is affected by a mosaic of regulatory factors. At any time point, one or more of these factors are themselves differentially regulated, globally by hormones, or individually on the transcriptional, post-transcriptional, translational or post-translational levels. Thus, Cx43 regulation by miRNA is not a static process, but is intricately and dynamically linked with other cellular pathways. Arrows designate regulatory processes that can have an effect on activity levels of others.

4.4 Expression of breast cancer-associated miRNA likely depends on its stage and subtype

This project focused on the role of miRNA in normal GJIC and mammary gland development; however, potential role of these molecules in breast cancer progression can be seen by comparing expression of miRNA in T-47D cells, a luminal breast cancer cell line, with normal mammary glands. If miRNA that are normally tumour suppressors, meaning that their under-expression positively regulates progression, are downregulated in breast cancer cells, such miRNA could be contributing to the disease properties. On the other hand, if miRNA known to be oncogenic, meaning that their overexpression positively affects progression, are downregulated in breast cancer cells, such miRNA could also be having an effect on disease properties. If an aberrantly expressed miRNA also targets Cx43, then it might contribute to abnormal GJIC seen in many cancers (Holder et al. 1993; Cronier et al. 2009; Aasen et al. 2017; Busby et al. 2018). Examples of miRNA that showed differential expression in mouse mammary glands vs. breast cancer cells are shown in the table below.

Table 4.1: Examples of miRNA Showing Differential Expression in Mouse Mammary Glands vs. Breast Cancer Cells.

Up/down indicates expression above or below 500 RPM, in breast cancer cells vs. mammary glands, respectively. Literature demonstrating a role of the miRNA in cancer, if available, is also presented. If differential expression of the miRNA is associated with a certain stage of development in particular, that stage is indicated in the 'stage' column. 'Both' signifies that literature evidence is present for both up- or down-regulation of the miRNA; thus, the miRNA expression may depend on the cell type and stage of breast cancer (see text). *This miRNA was expressed at 501 RPM in the mammary gland, and above 15000 in breast cancer cells.

miRNA	*In vitro* vs. *in vivo* expression (up/down)	Regulation in Literature (up/down) + study	Stage
miR-141	Downregulated	Both (Choi et al. 2016; Taha et al. 2020; Finlay-Schultz et al. 2015)	Lactation
miR-200a-5p	Upregulated	Downregulated (Zeng et al. 2019)	Lactation
miR-199a-3p, miR-199a-5p	Downregulated	Both (Li et al. 2015; Li et al. 2016b; Shatseva et al. 2011)	N/A
miR-221-3p	Downregulated	Upregulated (Liang et al. 2018)	N/A
miR-92a-3p*	Upregulated	Upregulated (Yu et al. 2022; Jinghua et al. 2021; Cun et al. 2018)	N/A

4.4.2 miR-200 family and breast cancer

Some studies show increased miR-141 in breast cancer relative to normal tissue, whereas other articles find that it is downregulated. For this reason, miR-141 expression in breast cancer likely depends on the subtype or stage, as does Cx43 expression (Busby et al. 2018). For example, it has been shown that higher level of miR-141 is positively associated with tumour invasive and migratory characteristics, and negatively associated with growth, in triple negative breast cancer cells (Choi et al. 2016). Other studies have shown that miR-141 downregulation accounts for breast cancer cell properties (Finlay-Schultz et al. 2015). In our study, miR-141 was downregulated in T-47D cells compared with regular mammary glands at all stages, but especially lactation. Downregulation could be expected, as T-47D cells are less aggressive than highly invasive cell lines such as MDA-MB-231.

In contrast, other miR-200 family members were actually upregulated in this study, despite being more characteristic to aggressive cell lines. For example, our study found that miR-200a-5p was upregulated relative to normal mammary glands. Few studies exist on this miRNA, but one study indeed showed that suppressed miR-200a-5p expression can occur due to a complex between FEN1, PCNA and DNMT3a that mediates methylation of its promoter, resulting in increased proliferation of breast cancer cells (Zeng et al. 2019).

Some miRNA regarded as tumour suppressors or oncogenes were not expressed in mammary glands but not breast cancer cells, or vice-versa, in this study. For example, miR-200c has been proposed as an oncogenic miRNA as it is often overexpressed in breast cancer tissue and is associated with metastasis (Zhang et al. 2017). Ming et al. 2015a found that Cx43 overexpression resulted in increased migration of breast cancer cells, which was countered by miR-200a-3p overexpression. Our study did not identify those two miRNA as expressed in T-47D cells but not mammary glands, or vice versa. Differing findings of various studies re-affirm the complexity of links between Cx43 and breast cancer, which depend on the stage and subtype (Busby et al. 2018).

4.4.3 miR-199 family and breast cancer

In our study, miR-199-3p was downregulated in T-47D cells compared with normal mammary glands, at all of the key stages tested. miR-199a-3p and miR-199a-5p are overexpressed in some

cell lines, and underexpressed in others, suggesting that their role depends on the stage and subtype of breast cancer. For example, miR-199-3p was underexpressed and associated with metastasis in MDA-MB-231, CAL120, and HCC1395 cells (Li et al. 2015b), but in another study, overexpression of miR-199a-5p decreased invasive characteristics (Li et al. 2016b). On the other hand, increase in miR-199a-3p has also been associated with increased cellular proliferation (MDA-MB-231 and MT-1 breast cancer cells) (Shatseva et al. 2011). Again, this shows that miRNA can play dual functions as tumour suppressors or oncogenes in different conditions.

4.4.4 miR-26a family and breast cancer

miR-26a is a functional suppressor of proteins involved in cell cycle progression (Huang et al. 2019), and was expressed above 300000 reads per million in T-47D cells, as opposed to only around 5000 in the regular mammary glands. Thus, it is possible that the high expression of this miRNA accounts for the cancer properties (for example, resistance to cell cycle checkpoints) of T-47D cells. Some Cx43-related miRNA for which evidence exists showing their tumour-suppressive or oncogenic properties did not display aberrant expression in T-47D cells, such as hsa-miR-125b-5p, hsa-miR-222 and hsa-miR-101 (Loh et al. 2019; Singh et al. 2013), re-affirming that miRNA regulation of breast cancer properties is complex, and dependent on the stage and subtype. Further analyses will be required to identify aberrantly expressed miRNA, as this analysis only considered expressed/non-expressed as a parameter.

4.5 RISC loading specificity – a factor affecting Cx43 and GJIC properties in mice vs. humans?

During miRNA maturation, the double stranded pre-miR is loaded into Ago1, forming the RISC complex. Normally, the miR star (miR*) arm of the precursor is then selectively excised and degraded, leaving the miR strand (miR) to perform mRNA suppression. However, dual-fate miRNA are also documented whereby both miR or miR* strands have similar loading into Ago1 (Reichholf et al. 2019). The binding stability of base pairs at of the end of the double stranded miRNA precursor affects this process (Schwarz et al. 2003; Khvorova et al. 2003). When comparing expression of miRNA originating from the same precursor, strand selection was usually consistent between mouse and human; in other words, expression of 3p and 5p versions of the miRNA did not change. However, some particularly important regulators of Cx43 changed drastically in which mature miRNA were detected in miRNA-seq experiments. For example, miR-

200a-3p, a widely studied regulator of Cx43 (Ming et al. 2015a; Renthal et al. 2010), was elevated in addition to miR-200a-5p in human cells, but only miR-200a-3p was expressed in the mouse. This affects Cx43 expression, as different proportions of miR-200a-3p are present, and dual-fate miRNA typically have higher half-lives (Reichholf et al. 2019). Thus, species-specific GJIC regulation by miRNA might not be limited to differential miRNA gene transcription, but could include specificity of RISC complex loading. Unfortunately, inter-species differences in specificity of RISC complex loading, and the comparative effects on genes and organism characteristics, is minimally studied. This pattern was observed in many other important miRNA, such as miR-200b, miR-30a and miR-30e. Further analyses will be required to count single and dual-fate miRNA in our *in vivo* study to confirm if RISC loading specificity is a process differentiating miRNA regulation of GJIC in mice and humans.

4.6 miRNA transfections in T-47D cells

4.6.1 hsa-miR-125b-5p description

hsa-miR-125b-5p was chosen for validation of the functional effects of miRNA on Cx43. Firstly, hsa-miR-125b-5p has a bioinformatics-predicted binding site in Cx43 mRNA (TargetScan Mouse 8.0), and was shown to negatively regulate Cx43 expression in a previous report (Jin et al. 2013). However, on the other hand, *in vivo* sequencing data showed minimal mmu-miR-125b-5p at lactation, where luminal Cx43 is lowly expressed (Dianati et al. 2016). In other words, one line of evidence suggests that miR-125b-5p would inhibit Cx43 expression, whereas the second suggests that miR-125b-5p mirrors Cx43 expression, and may positively affect its expression. In addition, this miRNA was associated with both estradiol and prolactin exposure, and was one of the lesser studied miRNA in literature. For these reasons, hsa-miR-125b-5p was an interesting candidate to exogenously alter in T-47D cells.

4.6.2 Transfection methods

A mimic and inhibitor for hsa-miR-125b-5p were transfected in T-47D cells, and after 48 h, cells were harvested and RT-qPCR performed to measure their effect on Cx43 mRNA. Co-exposures were included, whereby estradiol 10 nM or prolactin 200 ng/mL were pipetted into the media for the final 8 of 48 h, to test the effect of hormones on the up- or down-regulation of Cx43 by hsa-miR-125b-5p. In addition, random transfection sequences (mimic and inhibitor negative controls, from Thermo Fisher), as well as lipofectamine and untreated controls were included to ensure

that Cx43 mRNA regulation does not occur by an unpredicted mechanism. For each replicate, transfections were repeated three times, one without co-exposure, and the other two with the hormone co-exposures, yielding nine six-well plates and three replicates total. Prior to these experiments, transfections were carried out in T-47D cells, and up- or down-regulation of hsa-miR-125b-5p was validated by RT-qPCR.

4.6.3 Transfection result and future analyses

In some plates, suppression of Cx43 expression by hsa-miR-125b-5p occurred, suggesting possible regulation, but in others, no effect or a slight increase was observed. Due to these observations, the effect of hsa-miR-125b-5p on Cx43 appeared to be only marginal overall. With this result, no effects on Cx43 regulation by hsa-miR-125b-5p for estradiol and prolactin plates were recorded, other than their global up- and down-regulation of Cx43 mRNA expression, respectively. miRNA can have many different cellular targets, and it is possible that hsa-miR-125b-5p is only a secondary target of this transcript. In the same way, mRNA transcripts have many different miRNA binding sites, which results in regulation at different strengths for those different miRNA. In some control plates, lipofectamine increased Cx43 expression, possibly clouding effects of the mimic and inhibitor. As a continuation of this part of the project, a different miRNA should be transfected from our list of candidates, and a transfection time of 24 (preferred) or 72 h can be attempted as conditions. Plausible candidates from our list could include the miR-200 or miR-30 families.

4.7 Perspectives

4.7.1 Conclusion

This study adds knowledge on importance of miRNA, which regulate approximately one third of the proteome (Macfarlane et al. 2010), in mammary gland development. In contrast to other articles, this project analyzed the miRNA profile at many stages of mouse development. This led to identification of distinct miRNA clusters that shed light on their expression patterns in the broader context of development, rather than a snapshot at one stage. The data can be used by mammary gland researchers studying not only GJIC, but other cellular processes, as the method of miRNA-seq identifies the global miRNA profile. Studies confirming the expression of miRNA on the individual level are important because minimal articles on miRNA sequencing in the mouse mammary gland exist.

This project also builds on information on the effect of hormones on the miRNA profile in breast cancer cells. As opposed to most research focusing on one hormone, this study combined *in vivo* experiments with four *in vitro* hormone experiments, shedding light on the effect of hormones on the miRNA profile. Different conditions were selected to account for different physiological hormone concentrations and allow comparison to limited available literature.

4.7.2 Follow-up studies

Potential follow-up studies to this project are firstly, to confirm whether or not Cx43 mRNA alterations by miRNA lead to effects on Cx43 protein levels. This is relevant to test, as mRNA expression changes do not always translate to protein level changes, and it will ultimately be protein Cx43 that participates in GJIC. This would be accomplished by carrying out mimic and inhibitor transfections of T-47D cells and testing Cx43 expression by Western blot. Direct binding of miRNA to the 3' UTR using a luciferase assay should be carried out to acquire more direct evidence of Cx43 mRNA regulation by miRNA. Other miRNA on our list of preferred candidates for Cx43 regulation can be transfected, and tested for links with estradiol, progesterone, prolactin and oxytocin, and such links can be tested with other Cxs, to broaden knowledge on the effect of miRNA on GJIC. Finally, due to the fact that gap junctions are linked with other junctional proteins, it would be interesting to take candidates for Cx43 regulation for which a 3' UTR site exists in other junctional proteins, and test for multiple junctional protein regulation by single miRNA.

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