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Over-expression of miR-30b provokes mammary gland development defect

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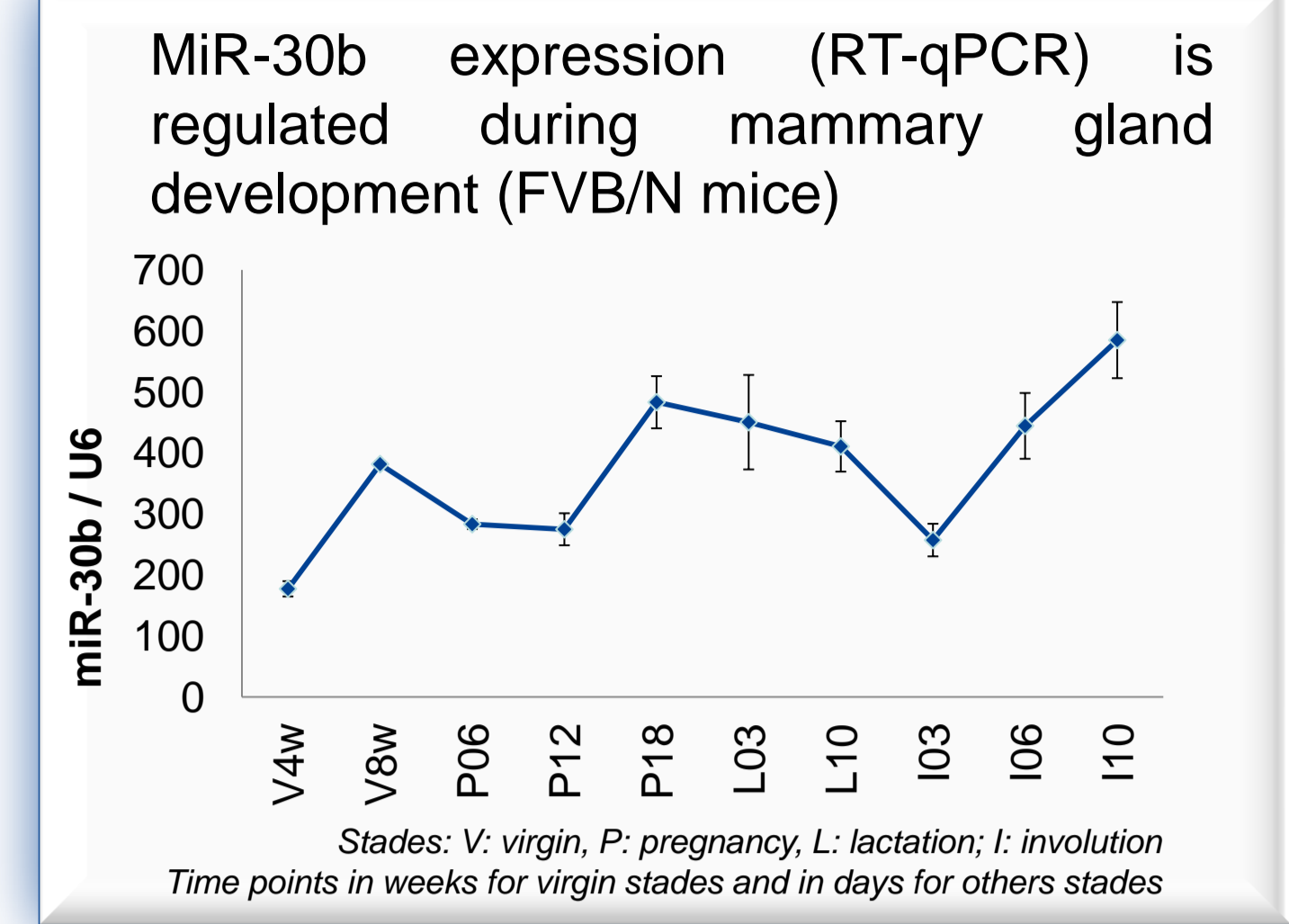


Introduction

MicroRNA (miRNA) are potential regulators of the normal mammary gland development and of the maintenance of mammary epithelial progenitor cells. In this study, we present the impact of the deregulation of one miRNA, miR-30b, on the establishment of a functional mouse mammary gland.

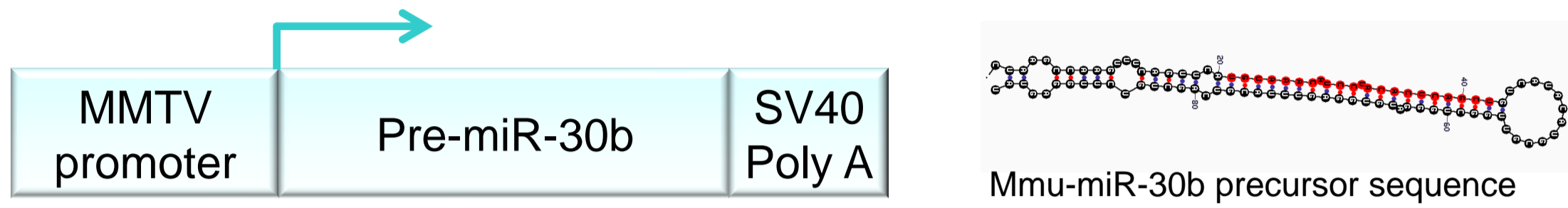
The miR-30 family, composed by 6 miRNA, is highly conserved in vertebrates. Its role has been suggested in the differentiation of adipocytes (Zaragosi *et al.*, 2011), B-cells (Lin *et al.*, 2011) and osteoblasts (Wu *et al.*, 2012), in the control of the pluripotency of embryonic stem cells (Zhong *et al.*, 2010) and of the epithelial-to-mesenchyme transition (EMT) (Joglekar *et al.*, 2009; Braun *et al.*, 2010; Zhang *et al.*, 2012). It is regulated by estrogen in breast cancer cells (Bhat-Nakshatri *et al.*, 2009), associated with mammary tumorigenesis and metastasis processes (Yu *et al.*, 2010), and it is one of the 13 miRNAs of which expression differs between inflammatory breast cancer (IBC) and non IBC (Van der Auwera *et al.*, 2010).

To investigate the role of miR-30b in the development and differentiation of the mammary epithelial cells, we have produced transgenic (Tg) mice over-expressing this miRNA and studied their mammary gland biology.



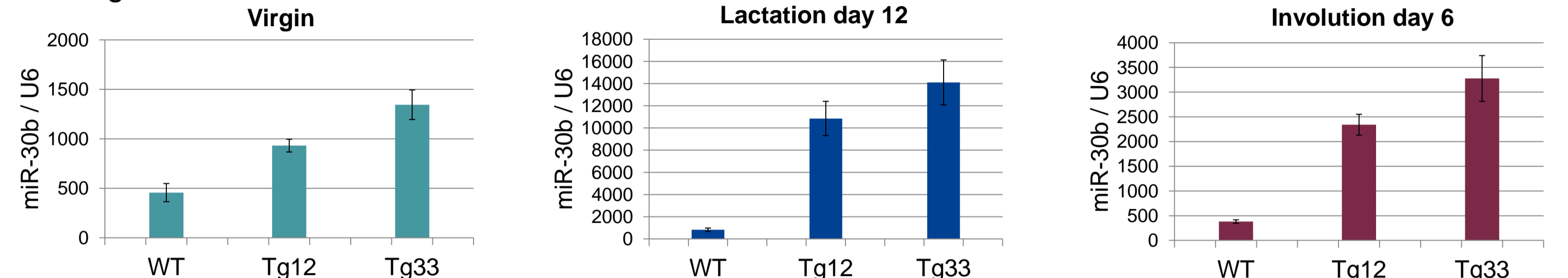
Mouse model

The mouse precursor of miR-30b was cloned under control of MMTV-LTR, enabling its over-expression from the early stages of the mammary gland development. This transgene was micro-injected in FVB/N eggs.



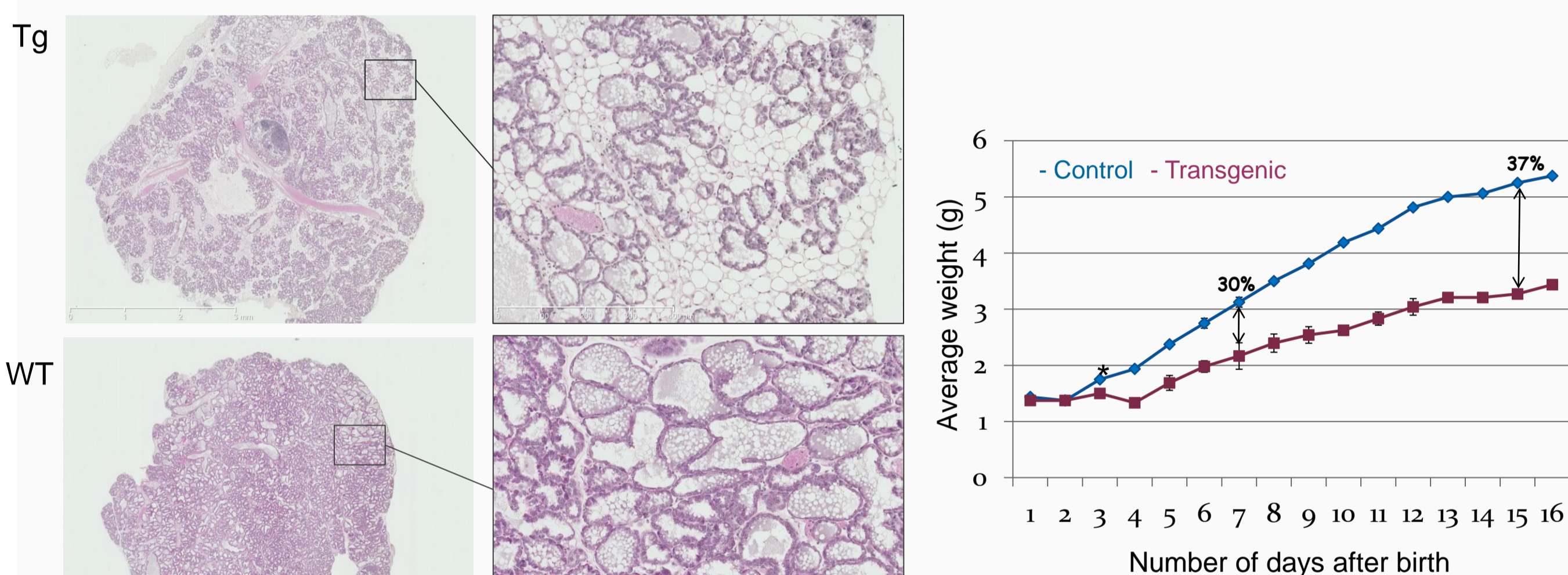
Among thirteen founders obtained, two were expressing the transgene and transmitted it to their progeny: lines Tg12 and Tg33.

The mammary miR-30b expression (RT-qPCR) is significantly higher (p -value $< 0,05$) in transgenic (Tg12 and Tg33) compared to wild type (WT) mice. This over-expression is more pronounced in lactation than virgin stage or during involution.



miR-30b over-expressing mice exhibit impaired mammary gland during lactation

While no noticeable phenotype was detected on mammary glands of virgin and pregnant mice, or during early lactation (day 3), an impairment of mammary gland development was observed from mid-lactation.



Mammary histological analyzes of Tg mice showed a **reduction of the epithelial tissue and of the alveolar filling** at lactation day 12 (Hematoxylin-eosin-saffron staining)

This phenotype was associated with a **significant growth defect of pups fed by Tg females**, that is reproduced in second lactation (data not shown).

Transcriptome analyzes performed using Affymetrix® Mouse Gene 1.1 ST arrays allowed to highlight mammary signaling pathways in which miR-30b is involved during lactation (day 12):

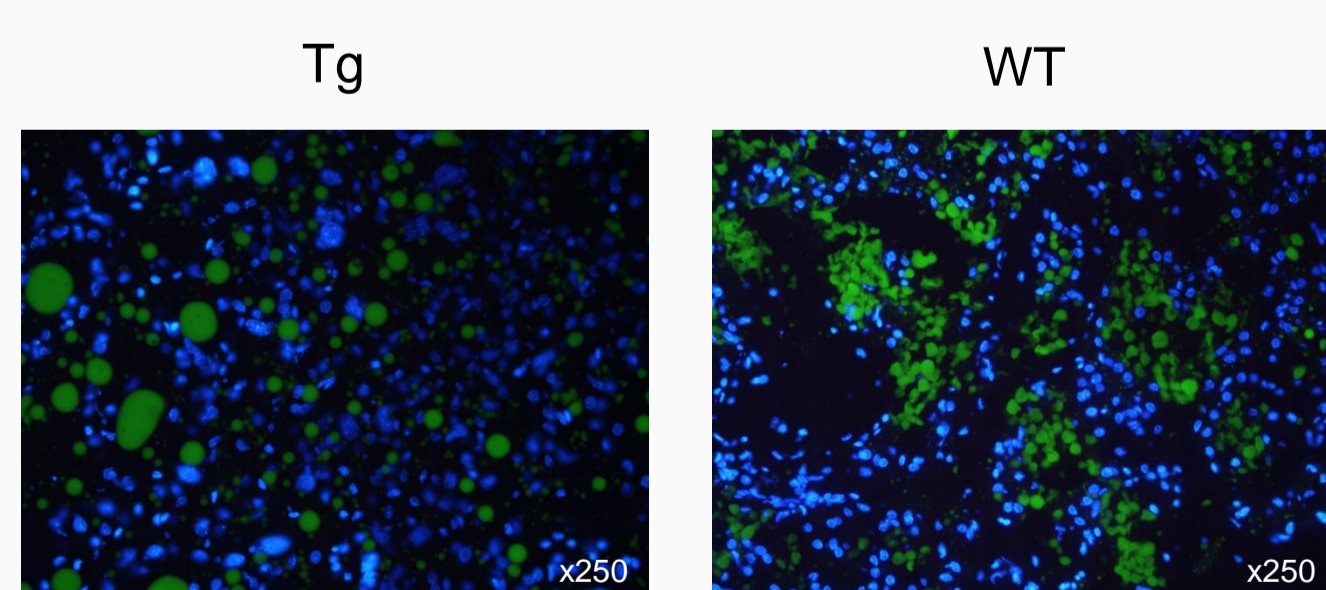
	Number of genes with adjusted p-value ≤ 0.05	Genes with adjusted p-value ≤ 0.05 and fold change $\geq 3 $
Down-regulated Tg vs WT	56	<i>Camk2b</i> , <i>D730048106Rik</i> , <i>Fmn1</i>
Up-regulated Tg vs WT	164	<i>9030619P08Rik</i> , <i>C1ca2</i> , <i>Gm10573</i> , <i>Ly6f</i> , <i>Pkd211</i> , <i>Slc16a4</i> , <i>Aox3</i> , <i>Gm1973</i> , <i>Mir30b</i> , <i>Saa1</i> , <i>Spp1</i> , <i>Apol6</i> , <i>Cldn4</i> , <i>Gm2897</i> , <i>Ncl2</i> , <i>Saa2</i> , <i>Tmem82</i> , <i>Bglap2</i> , <i>D830030K20Rik</i> , <i>Gm3002</i> , <i>Olf1328</i> , <i>Saa3</i> , <i>Tnfrsf23</i> , <i>Bglap-rs1</i> , <i>Dpyd</i> , <i>Gm5458</i> , <i>Orm1</i> , <i>Serp1nb8</i> , <i>Zbtb38</i> , <i>Ccnb1</i> , <i>Fam3c</i> , <i>Il1f9</i> , <i>Orm2</i>

The major network and functions identified with Ingenuity® Pathways Analysis (IPA) are the **cellular movement, the connective tissue development and function, the cell-to-cell signaling and interaction, and the organ development and tissue morphology.**

The expression of the classical epithelial markers, like Keratin 5, Keratin 8 or E-cadherin, did not differ between Tg and WT mice, which suggests that the number of epithelial cells itself is not affected.

Nevertheless, the **differentiation status of mammary epithelial cells** appears to be impacted, with a significantly differential expression of specific genes, in particular *Etf3*, *Erbp2*, *Erbp4* and *Spp1*/OPN.

The defect of pups growth could not be explain by a difference of the expression of genes coding for major milk proteins, which has not been detected. However, **the structure of the lipid droplets is abnormal** in Tg compare to WT (Nile Red (green) and Dapi (blue) staining, lactation day 12).

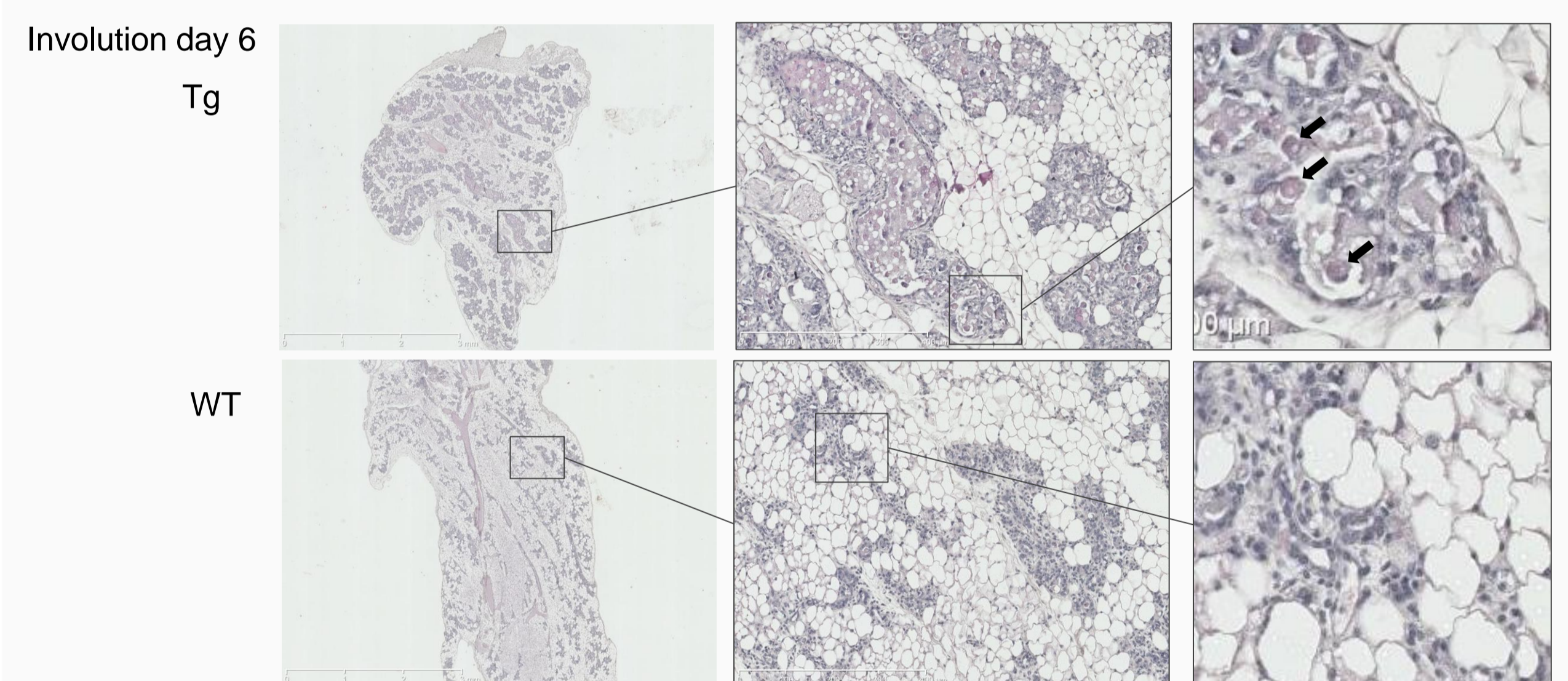


Moreover, many identified genes are **related to cell death** (*Casp6*, *Ccdc80*, *Cclca2*, *Cldn1*, *Cldn4*) and **acute phase response** (*Orm1*, *Orm2*, *Saa1*, *Saa2*, *Saa3*), which are typically expressed at the involution stage.

miR-30b over-expression leads to delayed mammary gland involution

During normal mammary gland development, the expression of miR-30b decreases at the beginning of involution compared to the lactation period, then its expression gradually increases until the late stages of involution. The involution consists on successive processes: the apoptose of the secretory cells, the degradation and remodeling of the extracellular matrix, an influx of macrophages and the adipogenesis, accompanying the repopulation of the mammary stroma by adipocytes.

The impact of the over-expression of miR-30b on mammary gland involution was explored and histological analysis showed that **some differentiated structures persist during involution** (days 3 and 6) and **apoptotic cells shed into the lumen are still evident** (arrows), suggesting a delay in the process in Tg mice. However, at day 11 of involution, there are no more differences between Tg and WT mice.



The transcriptome analysis performed at involution day 6 confirmed the histological observations. Top networks and functions identified by IPA® analysis are **the cell cycle, the cellular assembly and organization, the cellular function and maintenance, the cellular growth and proliferation, the inflammatory response, the lipid metabolism and the tissue morphology and development.**

	Number of genes with adjusted p-value ≤ 0.05	Genes with adjusted p-value ≤ 0.05 and fold change $\geq 3 $
Down-regulated Tg vs WT	209	<i>Acot11</i> , <i>Kcnk3</i> , <i>Cidea</i> , <i>Ntrk3</i> , <i>Fabp3</i> , <i>Tuba8</i> , <i>Inmt</i>
Up-regulated Tg vs WT	816	<i>4930578G10Rik</i> , <i>Clec4d</i> , <i>F7</i> , <i>Mmp13</i> , <i>Reg1</i> , <i>Spon1</i> , <i>BC055004</i> , <i>Clec4e</i> , <i>Il1f9</i> , <i>Mmp8</i> , <i>Saa1</i> , <i>Timp1</i> , <i>Ccl8</i> , <i>Clec5a</i> , <i>Il1m</i> , <i>Ms4a4c</i> , <i>Saa3</i> , <i>Tm4sf19</i> , <i>Ccr1</i> , <i>Cpxm1</i> , <i>Itgam</i> , <i>Msr1</i> , <i>Serpine2</i> , <i>Vsig4</i> , <i>Cldn4</i> , <i>Dpep2</i> , <i>Mir30b</i> , <i>Muc4</i>

The **abnormal persistence of epithelial cells** at this stage of involution is emphasized by a significant overexpression of Keratin 8 and E-cadherin.

Many significant deregulated genes are known to **play a role during the irreversible stage of involution** (Clarkson *et al.*, 2004, Stein *et al.*, 2004), especially some genes coding for chemokines (*Ccl6*, *Ccl7*, *Ccl8*, *Ccl9*), immune cell antigens (*Cd80*, *Cd86*, *Lrp1*, *Ly86*), proteins involved in the acute phase response (*Saa1*, *Saa3*), of complement (*C1qa*, *C1qc*) or immune related (*Tgfb1*, *Myd88*, *Emr1*), and involved in cell (*Mcl1*) or cell death (*Casp1*, *Cidea*, *Phlda1*, *Tgfb1*).

In addition, the overexpression of matrix metalloproteinases (MMP3/Str1, MMP8, MMP12, MMP13 and MMP inhibitor TIMP1) indicates that **the mammary gland of Tg mice is still in full remodeling process**, although it should be more advanced on day 6.

The strong expression of genes involved in the **acute phase response**, particularly *Saa1* and *Saa3*, is consistent with the inflammation expected from the abnormal failure to remove unnecessary differentiated alveoli.

Conclusion

These results emphasize the important role of microRNA in the biology of the mammary gland and demonstrate that the deregulation of one miRNA can have a significant impact on it. Especially, miR-30b acts on mechanisms which control the maintenance of lactation, but also to trigger the involution process. Now, we focus our work on the identification of miR-30b targets, using bioinformatics analysis and a RNA-seq approach to compare mRNA present in the RISC complex, resulting from co-immunoprecipitation, in WT and Tg mice.