UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

NOTCH AND WNT SIGNALING INTERPLAY ON REGULATION OF EARLY EMBRYO DEVELOPMENT

MARIANA RAPOSO BATISTA

Orientadora: Doutora Maria Elisabete Tomé Sousa Silva
Coorientador: Professor Doutor Luís Filipe Lopes da Costa

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 30 de Outubro de 2020

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To my parents and grandmother.

Acknowledgments

A friend of mine once told me that doing a PhD was very much like a marathon, rather than a sprint. He was right. My PhD was long, strenuous, with many ups and downs and, although the finish line was always present in my mind, there were times when exhaustion threatened to make it unattainable. Fortunately, there is a key difference between a marathon and a PhD: in a PhD, although most of the work, sweat and tears are ours, our journey is made easier by people that help us along the way in many different ways.

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Title

Notch and Wnt signaling interplay on regulation of early embryo development

Abstract

Mammalian early embryo development requires action of a complex network of cell signaling pathways that coordinates cellular proliferation and differentiation events. Notch is a major regulator in embryonic and adult scenarios, also interplaying with other signaling pathways, such as Wnt.

The objective of this work was to determine Notch signaling status in early embryo development and its influence on cellular differentiation and pluripotency maintenance, and on embryo competence to implant and develop to term. Additionally, the Notch/Wnt interplay was investigated in this scenario. Firstly, we analyzed individual embryo transcription of Notch components and their relation with transcription of pluripotency and differentiation gene markers (*Sox2*, *Oct4*, *Klf4*, *Cdx2*). Secondly, a pharmacological approach was used to induce Notch signaling (recombinant JAGGED1 and 2) and to inhibit Notch and/or Wnt signaling (DAPT and/or DKK1, respectively). Finally, embryos treated with DAPT and/or DKK1 were transferred to recipient females and implantation competency (at Day5 of gestation) and development to term (Day18) were evaluated.

Results showed that transcription of *Notch1-2*, *Jagged1-2* and *Hes1* was highly prevalent and dynamic along stages of development. Transcription of *Notch1*, *Notch2*, *Jagged2* and *Hes1* correlated with each other and with that of *Sox2*, *Oct4*, *Klf4* and *Cdx2*. *In vitro* embryo culture supplementation with JAGGED1 had no effect on embryo developmental kinetics, whereas supplementation with JAGGED2 abolished *Jagged1* transcription, downregulated *Cdx2* transcription and inhibited blastocyst hatching. Notch and Wnt had opposing effects on developmental kinetics, as Notch blockade retarded development and hatching, while Wnt blockade fastened it. We found evidences of Notch and Wnt interplay in early embryos as double blockade produced more severe phenotypes than expected by cumulative effects of single blockades. Notch and double blockade altered trophectoderm cell numbers and inner cell mass to trophectoderm ratio and all blockades altered transcription of *Sox2*, *Oct4*, *Klf4* and *Cdx2* throughout development. Implantation was unaffected by treatment, but Notch and double blockades affected the rate of Day18 developed fetuses. Notch blockade produced lighter and Wnt blockade heavier fetuses.

Overall, results indicate that Notch is active in early embryo development where, together with Wnt, plays a significant role in controlling the pace of differentiation and proliferation of the blastocyst, ultimately affecting development to term.

Keywords: Notch, Wnt, embryogeneis, pluripotency, differentiation

Título da tese

As vias de sinalização Notch e Wnt na regulação do desenvolvimento embrionário precoce

Resumo

O desenvolvimento embrionário precoce em mamíferos requer a ação coordenada de eventos de proliferação e diferenciação celulares. A correcta coordenação destes eventos está dependente de uma complexa rede de vias de sinalização intercelular. Uma das vias de sinalização intercelular mais conservadas em metazoários é a via Notch. Esta é responsável pela organização da diferenciação celular e manutenção da pluripotência em vários tecidos, quer na embriogénese quer na vida adulta, e interage com outras vias de sinalização, tal como a via Wnt, para este fim.

O objetivo deste trabalho foi a determinação da presença e atividade da via Notch no desenvolvimento embrionário precoce em embriões de murganho entre os 3.5 e os 4.5 dias *post coitum* (dpc). Adicionalmente procurou saber-se quais os elementos da via responsáveis pela transdução de sinal e se a sinalização Notch atua em conjunto com a via Wnt neste cenário. Finalmente procurou estabelecer-se a existência de relações entre os elementos da via Notch e marcadores de estados de pluripotência (*Sox2*, *Oct4*, *Klf4*) e diferenciação (*Cdx2*) embrionários, assim como a possível influência das vias Notch e Wnt na capacidade de implantação embrionária e no desenvolvimento fetal até termo.

A transcrição dos componentes da via Notch (recetores *Notch1-4*; ligandos *Delta-like1* e *4* e *Jagged1-2*; e efetores *Hes1-2*) e dos marcadores *Sox2*, *Oct4*, *Klf4* e *Cdx2* foi analisada em embriões individuais e inteiros recorrendo à técnica de PCR em tempo real. De seguida, foi usada uma abordagem farmacológica *in vitro* para induzir a via Notch com proteínas recombinantes JAGGED1 e JAGGED2, e para inibir as vias Notch e Wnt com DAPT (um inibidor da gama-secretase) e/ou DKK1 (um inibidor competitivo da via), respectivamente. Os embriões foram recolhidos *in vivo* aos 2.5 dpc e postos em cultura *in vitro* até aos 4.5 dpc com os respetivos tratamentos. Desta forma foram analisados a cinética de desenvolvimento embrionário dos 3.5 aos 4.5 dpc, a contagem diferencial de células da trofectoderme (TE) e do botão embrionário (ICM), e a transcrição de genes das vias Notch e Wnt e dos marcadores de pluripotência/diferenciação em embriões de 3.5 dpc. Finalmente, blastocistos e blastocistos expandidos tratados com DAPT e/ou DKK1 foram transferidos aos 4.0 dpc para fêmeas recetoras e analisaram-se as taxas de implantação aos 5 dias de gestação e a taxa de desenvolvimento fetal aos 18 dias de gestação, assim como os pesos dos fetos resultantes.

Os resultados mostraram que a transcrição dos genes *Notch1-2*, *Jagged1-2* e *Hes1* é altamente prevalente e dinâmica ao longo do desenvolvimento embrionário precoce. Pelo contrário, a transcrição de *Notch3-4*, *Dll4* e *Hes2* é inconstante nos embriões, independentemente do seu estadio. As transcrições de *Notch1*, *Notch2*, *Jagged2* e *Hes1* estão correlacionadas entre si e correlacionam-se com as de *Sox2*, *Oct4*, *Klf4* e *Cdx2*. A suplementação do meio de cultura com JAGGED1 não teve efeito na cinética de desenvolvimento embrionário nem na transcrição dos genes analisados, enquanto que a suplementação com JAGGED2 aboliu a transcrição de *Jagged1*, diminuiu a transcrição de *Cdx2* e inibiu a eclosão dos blastocistos. Notch e Wnt mostraram efeitos opostos na cinética de desenvolvimento embrionário: o bloqueio da via Notch atrasou o desenvolvimento embrionário dos 3.5 aos 4.5 dpc e a sua eclosão, enquanto que o bloqueio da via Wnt acelerou-os. Verificámos ainda que existe uma interação entre as vias Notch e Wnt nesta fase de desenvolvimento visto o bloqueio de ambas as vias promover fenótipos mais severos do que o esperado pelos efeitos cumulativos dos bloqueios de cada via individualmente. O bloqueio da via Notch e o duplo bloqueio alteraram o número de células da trofectoderme e o rácio ICM:TE, e todos os bloqueios alteraram as transcrições de *Sox2*, *Oct4*, *Klf4* e *Cdx2* ao longo do desenvolvimento. A implantação aos 5 dias não foi afectada pelos tratamentos. No entanto, o bloqueio da via Notch e o duplo bloqueio diminuiram a taxa de fetos desenvolvidos a termo. Adicionalmente, o bloqueio de Notch produziu fetos mais leves, enquanto que o bloqueio de Wnt produziu fetos mais pesados do que os fetos do grupo controlo.

Em conclusão, os resultados indicam que a via Notch está ativa no desenvolvimento embrionário precoce e que, em conjunto com a via Wnt, é um importante regulador da cinética de desenvolvimento embrionário e de eventos de proliferação e diferenciação celulares. Podemos ainda concluir que as ações das vias nesta fase precoce do desenvolvimento têm consequências, não só a curto prazo, mas também a longo prazo na capacidade de desenvolvimento fetal a termo.

Palavras-chave: Notch,Wnt, embriogénese, pluripotência, diferenciação

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LIST OF ABREVIATIONS

ΔCt – delta threshold cycle

ΔΔCt – delta-delta threshold cycle

ANCOVA – analysis of covariance

ANOVA – analysis of variance

ART – assisted reproductive techniques

BL – blastocyst

bp – base pair

BSA – bovine serum albumin

cDNA – complimentary deoxyribonucleic acid

CEBEA – Comissão de Ética e Bem-Estar Animal

CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal

CM – compact morulae

Ct – threshold cycle

DAPT – N-S-phenyl-glycine-t-butyl ester

DGAV – Direção Geral de Alimentação e Veterinária

DLL1 – delta-like 1

DLL4 – delta-like 4

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

dpc – days *post coitum*

EBL – expanded blastocyst

EDTA – diaminoethane tetraacetic acid

EGA – embryonic genome activation

eHBL – early hatched blastocyst

EPI – epiblast

ES – embryonic stem

FCT – Portuguese Foundation for Science and Technology

FMV – Faculdade de Medicina Veterinária

HBL – hatched blastocyst

hCG – human chorionic gonadotrophin

HES – hairy/enhancer of split

Hprt1 – hypoxanthine phosphoribosyltransferase 1

ICC – immunocytochemistry

ICM – inner cell mass

IgG – immunoglobulin G

iPS – induced pluripotent stem

IVF – *in vitro* fertilization

KSOM – potassium-supplemented simplex optimized medium

LRP5/6 – low density lipoprotein receptor-related proteins 5/6

MET – maternal to embryo transition

MGA – mid-preimplantation gene activation

miRNA – micro ribonucleic acid

mRNA – messenger ribonucleic acid

NECD – Notch extracellular domain

NICD – Notch intracellular domain

NTC – no-template control

PBS – phosphate-buffered saline

PCP – Planar Cell Polarity

PCR – polymerase chain reaction

PrE – primitive endoderm

qRT-PCR – quantitative real-time polymerase chain reaction

RBPJ – recombination signal binding protein for immunoglobulin kappa J region

RNA – ribonucleic acid

Rps29 – ribosomal protein S29

RT-PCR – reverse transcriptase polymerase chain reaction

s.e.m. – standard error of the mean

sc-RNAseq – single cell ribonucleic acid sequencing

siRNA – small interfering ribonucleic acid

SPI – single plane image

TCF/LEF – T cell factor/lymphoid enhancer factor

TE – trophectoderm

TGFβ – transforming growth factor beta

Tm – melting temperature

ZP – zona pellucida

1. INTRODUCTION

Reproduction is key for the perpetuation of life as we know it and the study of animal reproduction, particularly mammalian reproduction, is of vital importance for humankind. Its understanding can aid in many aspects of our world's sustainability, namely to increase livestock reproductive efficiency to ensure a sufficient, safe and economically viable food supply (Granleese 2015), to preserve endangered species and natural habitats and control feral populations (Herrick 2019), and, in humans, to either control birth rate or to treat infertility (Mascarenhas 2012).

Most of animal and human pregnancy losses occur in the early embryo development stage (Diskin and Morris 2008; Jarvis 2016) and their importance is even more significant when dealing with economically valuable embryos as in the case of high genetic merit animals, or with human embryos. Also, since artificial reproduction techniques are fairly new, particularly in humans, its long term consequences in individuals born from such procedures is still not fully known (Wang and Sauer 2006) and unexpected effects of attempts to improve fertility need to be assessed.

Early embryo development is highly complex and events that lead to cellular differentiation and pluripotency maintenance need to be precisely orchestrated between embryonic cellular populations themselves and with maternal tissues. It is therefore logical that intercellular communication mechanisms are at play and deciphering their actions could prove useful in the attempt to improve embryo viability in cases of subcompetent embryos or adverse uterine conditions. Notch is a well conserved intercellular communication signaling pathway that has been shown to be present in embryos of various stages (Cormier et al. 2004; Wang et al. 2004; Adjaye et al. 2005; Aghajanova et al. 2012; Casanova et al. 2012). However, its role in the complex signaling network of early embryonic development remains elusive. The work here presented uses the mouse embryo model to address this knowledge gap. The hypothesis behind the studies here described was that Notch and Notch-Wnt signaling interplay are key regulators of the pace of early embryonic development, which could affect later fetal development. We confirmed the presence of Notch pathway components in early embryos and the activity status of Notch canonical signaling. Through *in vitro* pharmacological gain of function and inhibition strategies, we uncovered a role of Notch signaling and of Notch-Wnt interplay in embryonic cellular differentiation and pluripotency maintenance events. Finally, we shed light on the carry-over effects of these early signaling events on later embryo competence to implant and develop to term using embryo transfer experiments.

This thesis is comprised of a literature review that will aid in the interpretation of the following chapters, including a summary of the main early embryo development events, and a state of the art on Notch and Wnt intercellular signaling pathways and their known functions and interactions in several scenarios. Additionally, the studies here presented were converted into two manuscripts published or submitted for publication in peer-reviewed international journals, which comprise the two chapters of the experimental work. The first experimental chapter reports the confirmation of Notch presence and activity in early embryo development and its impact on embryo ability to develop and hatch *in vitro*. The second experimental chapter confirms the existence of interplay between Notch and Wnt signaling, which operate in opposing ways and in a developmental stage specific manner, to regulate the pace of early embryo developmental kinetics, pluripotency and differentiation events and ultimately embryo survival and development to term. Finally, a general discussion and a list of references are provided in the last chapters.

From the work presented in this thesis resulted the following:

1. **Notch and Wnt interplay in the regulation of the pace of embryo developmental kinetics.**
   M Batista, P Diniz, A Torres, D Murta, L Mateus, L Lopes-da-Costa, E Silva (2016) Oral presentation. Proceedings of the 2016 ESDAR Conference, 27-29 October, Lisbon, Portugal (http://onlinelibrary.wiley.com/doi/10.1111/rda.12800/full).

2. **Notch signaling in mouse blastocyst development and hatching.**
   MR Batista, P Diniz, A Torres, D Murta, L Lopes-da-Costa, E Silva (2020).
   BMC developmental biology, 20(1), 9.

3. **Early embryo balanced Notch and Wnt signaling interplay is required for embryo and fetal development to term.**
   MR Batista, P Diniz, D Murta, A Torres, L Lopes-da-Costa, E Silva
   Submitted to *Reproduction*.

2. LITERATURE REVIEW

Embryo development has been the focus of scientific studies for many years. Early studies have mainly used non-mammalian species as animal models, such as *Drosophila* (of the Arthropoda phylum) and *Xenopus* and zebra fish (of the Chordata phylum). These studies have largely contributed to our knowledge in cellular mitotic cycles and cellular differentiation and migration mechanisms, which are all necessary for the progression of one cell to a fully formed adult organism.

Despite the invaluable knowledge gained by the study in these models, it does not exactly mirror all embryonic development events in mammals. The study and manipulation of mammalian embryonic development proved to be more difficult due to the size of the "eggs", which are small comparing to other animals; their lesser availability, since one individual produces far less offspring at one given time than other non-mammalian species, and finally, because mammalian embryos naturally develop inside the maternal organism, which specific conditions have only recently been satisfactorily mimicked *in vitro* (Gilbert 2000). This work focused on mammalian embryo development, using the mouse model, hopefully allowing the obtained knowledge to improve assisted reproductive techniques (ART) in both human and animals.

2.1. Early embryo development

The events of early embryonic development following fertilization, such as successive cleavages up to the morula stage (8-32 cells), compaction of embryonic cells – blastomeres – and formation of the blastocyst, as well as cellular differentiation events, are conserved phenomena among mammalians (Betteridge and Rieger, 1993), although the speed to which they happen depends on the considered species. In the mouse, early embryo development is fast compared to the bovine (Van Soom et al. 1992; Gordon 1994) or humans (Rodriguez-Osorio et al. 2012) and the embryo is ready to implant at 4.5 days post-coitum (dpc) (Nagy et al. 2003).

Mammalian embryo development begins with the fertilization of an oocyte by a sperm cell inside the female oviduct. From the interactions between these two haploid cells, a fusion process occurs and a diploid totipotent cell is formed – the zygote (Evans 2002). This newly formed cell undergoes a cell division process called cleavage, where the cytoplasm of the original cell is divided by the two resulting cells – blastomeres, thus maintaining the overall volume of the embryo (Aiken et al. 2004; Casser et al. 2017). In the mouse, this first

cleavage starts 16-20 hours after fertilization and the following cleavages occur at 12 hour intervals (Marikawa and Alarcon 2012). After three cleavage cycles, at the late 8-cell stage, the blastomeres begin to attach firmly to each other, which involves cadherin-dependent filopodia (Fierro-González et al. 2013), and acquire apical-basal polarity, thus beginning the compaction process (Marikawa and Alarcon 2012; Schrode et al. 2013). From the 8-cell to 16-cell and 16-cell to 32-cell stages, blastomeres divide in two modes: symmetric (with the division plane perpendicular to the surface of the embryo) which will generate polar cells that maintain their outer position in the embryo; or asymmetric (with the division plane parallel to the surface of the embryo) which will generate one polar outer cell and one apolar inner cell (Johnson and Ziomek 1981). In this manner, the outer cells retain a polarized phenotype, while the inner cells lose their polar characteristics (Rossant and Tam 2009). An additional mechanism proposed by Yamanaka et al. (2010), who have tracked the origins of individual blastomeres from the 8-cell stage up to the blastocyst stage, considers that apolar cells will take up an inner position, regardless of their initial position after an outer blastomere cleavage, due to their intrinsic higher cortical tension. Currently, it is accepted that, at the morula stage, blastomeres take cues from both their surrounding cells and environment (Mihajlović and Bruce 2017).

The early differences in these two types of cells introduces the first cellular differentiation, where outer and polar cells will originate the trophectoderm (TE) which will contribute to the fetal portion of the placenta, and inner apolar cells will originate the inner cell mass (ICM), although at this stage there is still plasticity in cellular fate determination (Stephenson et al. 2010; Tarkowski et al. 2010; Toyooka et al. 2016).

After the fifth cleavage, between the 16- and 32-cell stage, the compact morula begins to develop into a blastocyst. In the blastocyst, the first two cellular lineages, ICM and TE, become definitively established and the previously observed plasticity of cellular fate is no longer observed (Suwińska et al. 2008). The formation of the blastocyst requires the creation and maintenance of a fluid filled cavity called blastocoel. The blastocoel begins as one or more microlumens that result from the exocytosis of intracellular vesicles formed in the outer cells that coalesce into a growing cavity (Marikawa and Alarcon 2012). The blastocoel further expands due to the generation of an osmotic gradient and consequent water influx from the exterior of the embryo. The osmotic gradient is accomplished by the accumulation of sodium ions (Na^+) in the intercellular space, which are actively transported by Na^+/K^+-ATPase enzymes localized in the basolateral membrane of TE cells (Betts et al. 1998; Houghton et al. 2003) and the influx of water is facilitated by numerous aquaporins also located in TE cellular membranes (Barcroft et al. 2003). Moreover, the TE has the vital role of maintaining a selective barrier between the blastocoel and the extraembryonic space.

This barrier is achieved by the paracellular sealing of TE cells by tight junctions (Eckert and Fleming 2008; Wang et al. 2008) and desmosomes (Fleming et al.1991). The blastocoel will thus continue to increase in size throughout preimplantation development, increasing overall embryo diameter.

After the first cellular differentiation, which originates the TE and ICM, there is a second lineage commitment in which ICM cells differentiate into the epiblast (EPI), which will originate all the tissues of the embryo itself, or into the primitive endoderm (PrE), a layer of epithelial cells that separates the EPI from the blastocoel and that will develop into the parietal and visceral endoderm of the yolk sac and as a subpopulation of cells in the early gut tube of the embryo (Kwon et al. 2008). It is believed that individual ICM cells are already predestined as future EPI or PrE cells in the early blastocyst and, as the blastocyst matures and expands, they become fully committed and move to their final location (Chazaud et al. 2006).

When the embryo is fully expanded and has reached the uterus, the hatching process takes place. This process follows the thinning of the zona pellucid (ZP) and its rupture is caused by a combination of the increase in embryo cell number and volume (Montag et al. 2000) and blastocyst and uterine produced enzymes (Montag et al. 2000; O'Sullivan et al. 2001). After the rupture of the ZP, the embryonic mass is released and the embryonic TE comes in direct contact with the endometrium.

2.2. Transcriptional regulators of cellular differentiation and pluripotency maintenance

As previously mentioned, early embryo development comprises two sequential cell fate decisions – TE and ICM from totipotent blastomeres, and EPI and PrE from ICM cells. These differentiation processes are accomplished by the reprogramming of cellular morphological and functional characteristics and are driven and maintained by a wide set of transcriptional markers. Robustness of the early cell lineage commitment is essential, as a change in one cell will be passed on to a large quantity of offspring cells and could endanger overall embryo development. Computational models have suggested that cell fate decisions are therefore dependent of auto-activating and mutual inhibiting clusters of transcriptional factors, rather than individual transcription factors (Sharifi-Zarchi et al. 2015). However, since in the same cluster there seems to be one or a small number of transcription factors that are master lineage indicators and others that have merely supporting roles over the former's

expression and function (Bergsmedh et al.2011; Sharifi-Zarchi et al. 2015), it is sufficient to refer to the main transcription factors when addressing early cell lineage commitment.

Among these transcriptional markers OCT4, SOX2 and KLF4 are known as the core transcription factors that are essential in the induction and maintenance of the pluripotent state of embryonic stem (ES) cells (which are derived from ICM cells) (Schmidt and Plath 2012). They are essential in assuring a correct preimplantation embryo development and can be used as pluripotency markers (Huang et al. 2015). Their combined actions lead to the expression of other pluripotency associated genes such as Nanog which is a homeodomain-containing protein essential in maintaining ICM and EPI cell populations in the embryo (Mitsui et al. 2003).

OCT4, also known as POU5f1, is a POU domain transcription factor that is expressed exclusively in blastomeres, pluripotent early embryo cells and in the germ cell lineage (Nichols et al. 1998; Pesce et al. 1998). The depletion of both maternal and embryonic *Oct4* transcripts promotes a developmental arrest at a wide range of embryonic stages before blastocyst formation (Tan et al. 2013). Studies in ES cells revealed that the tight regulation of OCT4 availability is essential for the maintenance of pluripotency states and that an excess or deficit results in cellular differentiation (Niwa et al. 2000). These observations were recently confirmed in preimplantation mouse embryos, where the maintenance of proper OCT4 expression levels is essential for correct embryonic development (Fukuda et al. 2016). At the morula stage, OCT4, which was weakly expressed until then, becomes strongly and equally expressed at the nuclei of all blastomeres (Palmieri et al. 1994; Pesce and Schöler 2000), as its early action is necessary for later TE specification (Emura et al. 2016). After blastocyst formation, OCT4 becomes restricted to the nucleus of ICM cells (Palmieri et al. 1994; Strumpf et al. 2005). In addition to its direct influence on cellular transcription programs, OCT4 also cooperates with a series of partners, namely the transcription factor Sry-related Sox-2 (SOX2), to activate these programs (Nishimoto et al. 1999).

SOX2 regulates transcription both by acting as a transcription factor and as a regulator of chromatin architecture (Scaffidi and Bianchi 2001). SOX2 expression overlaps OCT4 expression in the early development and is also found mainly in pluripotent lineages of the mouse preimplantation embryo (ICM and EPI) and germ cells (Avilion et al. 2003) as both transcription factors act in coordination to maintain the undifferentiated state of the ICM and EPI blastomeres and preventing their transformation into TE. SOX2 protein is primarily nuclear in all blastomeres at the 8-cell stage and in recently compacted morulae, although in the late morulae SOX2 staining becomes more diffuse in the outer cells (presumptive future TE) (Avilion et al. 2003). In blastocysts, this protein is mostly nuclear in the ICM cells and

cytoplasmic in TE cells (Avilion et al. 2003), although some TE cells can show some nuclear staining (Keramari et al. 2010). SOX2 null mutants only have discernible phenotypes after implantation, when EPI cannot preserve its pluripotent identity (Avilion et al. 2003). However, studies using siRNA to remove both maternal and zygotic mRNA showed that embryos fail to develop beyond the morula stage as they are unable to form the blastocoel (Keramari et al. 2010). Therefore, essentially maternally derived SOX2 acts as a regulator of *Cdx2* transcription among other TE associated transcription factors and markers in the morula, proving to be critical in TE formation at that stage, while not affecting *Oct4* transcription levels (Keramari et al. 2010).

KLF4 (also known as gut-enriched Krüppel-like factor4) is a zinc finger transcription factor that interacts directly with OCT4 and SOX2 in induced pluripotent stem (iPS) cells and ES cells to form a complex, which activates downstream targets required for cellular pluripotency maintenance such as Nanog (Wei et al. 2009). Transcription of *Klf4* is found early in preimplantation embryo development (Jiang et al. 2008; Guo et al. 2010) and its expression, as investigated in the mouse and rhesus monkey embryos from the morula to the blastocyst stage, although present in all blastomeres, becomes more intense in ICM cells in the blastocyst (Tang et al. 2011) and in EPI cells in the expanded blastocyst (Harvey et al. 2009; Dhaliwal et al. 2018). KLF4 knockout mouse embryos develop to term and die shortly after birth due to loss of skin barrier function (Segre et al. 1999). The survival of the embryo to term is possibly due to the redundancy with other KLF-family members (KLF2 and 5), as observed in embryonic stem cells (Jiang et al. 2008).

On the other hand, the first differentiation event is marked by the nuclear expression of the transcription factor CDX2 in TE cells. CDX2 is a caudal-related homeodomain protein that is a key regulator of the TE lineage as its expression in ES cells is sufficient to drive them to differentiate into trophoblastic cells (Niwa et al. 2005), being used as one of the earliest markers of TE differentiated cells. Embryos lacking CDX2 form blastocyst-like structures, however, they do not implant since their TE-like structure is not fully functional (Strumpf et al. 2005). Studies have shown that CDX2 is responsible for promoting TE development and repressing ICM lineage formation by repressing OCT4 expression through its interaction with the transcription factor BRG1 (Wang et al. 2010).

Up to the 8-cell stage all blastomeres are equally totipotent and have similar expression patterns of transcription factors (Guo et al. 2010). At the 8- to 16-cell transition the first differentiated cell lineages begin to develop, although not irreversibly, through stochastic processes and/or asymmetric cell divisions (Dietrich and Hiiragi 2007). In fact, at this stage, all blastomeres equally express CDX2 (Strumpf et al. 2005), OCT4 (Palmieri et al.

1994; Pesce and Schöler 2000), SOX2 (Keramari et al. 2010) and KLF4 (Harvey et al. 2009; Dhaliwal et al. 2018).

At the beginning of compaction the epithelial identity of the outer cells begins to form and their apical domain becomes enriched with the PARD6B protein, establishing a normal epithelial morphogenesis (Alarcon 2010). This epithelial identity drives the activity of CDX2 in future TE cells, marking one of the earliest events of TE specification (Ralston and Rossant 2008). CDX2 directly binds to OCT4 promoter to repress *Oct4* and *Nanog* transcription, which up to this point was ubiquitous in all blastomeres (Niwa et al. 2005). Conversely, in future ICM cells, CDX2 expression is repressed (Loh et al. 2006) and OCT4 expression is maintained (Chew et al. 2005) by the complex formed by OCT4 and SOX2. It is the decrease in mRNA of pluripotency associated transcriptions factors and not the increase in differentiation transcription factors that drives lineage segregation from the morula to the blastocyst stage (Guo et al. 2010). Although at the late compaction stage some blastomeres still present both types of transcription factors independently of their position, as development progresses and the blastocyst is formed, the outer cells exhibit increasingly higher expression of TE specific transcription factors, and inner cells exhibit increasingly higher expression of ICM specific transcription factors, until both populations are perfectly segregated (Schrode et al. 2013).

At the second cellular differentiation that originates the EPI and PrE, pluripotency associated transcription factors such as OCT4, SOX2 and Nanog are present in both cell types at the blastocyst stage. It is at the late blastocyst stage (of about 64 cells) that Nanog becomes restricted to EPI cells and GATA6 to PrE cells and both cellular populations become spatially and transcriptionally separated (Plusa et al. 2008).

2.3. Maternal to embryo transition

Immediately after fertilization the newly formed zygote is transcriptionally silent and the very first cellular processes are dependent on maternal stored mRNAs and proteins. A major event that ensures embryonic competence to develop is the embryo's ability to transcribe and translate its own genome. This shift in control of cellular functions from the mother to the embryo is called the maternal to embryo transition (MET) (reviewed by Schultz 2002). The timing of MET is variable among species, and can be as early as the 2-cell stage in the mouse and 4-cell stage in humans, or as late as the 16-cell stage in rabbits, although the most frequently found in domestic species is the 8-cell stage as found in cow, sheep and pig (reviewed by Telford et al.1990).

MET is characterized by the degradation of oocyte derived mRNA (Hamatani et al. 2004) and proteins (Tsukamoto et al. 2008) and by the embryonic genome activation (EGA) (reviewed by Bell et al. 2008). Although the timing of MET and EGA is variable among species, the pattern by which it happens is fairly constant. Many studies in the mouse have observed that EGA is performed in waves. Abe et al. (2015) confirmed the existence of a first minor wave of transcription at the 1-cell stage, which is uncoupled from an efficient production of functional mRNAs. This wave is followed by a major wave of transcription that peaks at the 2- to 4-cell stage, which is thought to be crucial to prepare basic cellular machinery (Hamatani et al. 2004; Abe et al. 2015). A second major wave of transcription occurs at the 8-cell stage and is called "mid-preimplantation gene activation" (MGA). It is believed that these transcripts may code for key regulators of future TE and ICM differentiation (Hamatani et al. 2004).

Despite the degradation of maternally derived mRNA proteins and the intense *de novo* embryonic transcription and translation, it is though that up to 10% of maternal mRNA persists until the blastocyst stage (Bachvarova and De Leon 1980), as well as many maternally derived proteins, which are essential in TE and ICM segregation (Ohsugi et al. 2008; Li et al. 2008).

2.4. Implantation

Implantation is essential for the successful development of the mammalian fetus to term and is believed to be one of the major limiting steps of *in vitro* fertilization (IVF) protocols (Dekel et al.2014). It requires a complex crosstalk between the embryo and the maternal endometrium and a precise synchronization of embryo acquisition of competency to

implant and maternal endometrium receptivity. The uterus is only receptive to embryo implantation for a limited time frame called the "maternal window of implantation", which in the mouse is as short as one day (reviewed by Zhang et al. 2013).

After the embryonic mass escapes from the ZP, TE cells evolve into the trophoblast (reviewed by Sutherland 2003), which specializes in establishing a direct contact with the endometrium to form the placenta and begin to collect the necessary nutrients and oxygen to continue embryo development. However, the implantation process is variable among species. Ungulate (such as ruminants and horses) hatched embryos remain disconnected from the uterus during a phase of rapid trophoblast growth, known as elongation, which is concurrent with gastrulation (Blomberg et al. 2008). During this stage, the embryo's trophoblast does not invade the uterine epithelium and only creates finger-like papilla that project into the lumen of uterine glands (reviewed by Gonzales et al. 1996). Placentation itself occurs later and is of an epitheliochorial type. On the other hand, the mouse implantation process is very different from ungulate implantation and resembles more closely what happens in humans and other primates, since they have a haemochorial type of placentation. Here, following hatching, the trophoblast cells actively invade and cross the basement membrane of the uterine epithelium to gain access to the stromal bed, accessing the maternal blood circulation and resources (reviewed by Carson et al. 2000).

2.5. Intercellular signaling pathways in early embryos

As previously described, the developing early embryo relies on well orchestrated events of cellular proliferation and differentiation processes to ensure its progression to a fully functional animal. This orchestration in the early embryo is achieved not only through maternal support and paracrine regulation, as seen by the adaptation of oviductal secretions to the presence of embryos (Lee et al. 2002; and reviewed by Li and Winuthayanon 2017) and the uterine receptivity during the maternal window of implantation (reviewed by Zhang et al. 2013); but mostly from intrinsic embryonic intercellular communication that interprets spatial, polarity and environmental clues.

Many studies have identified a wide variety of intercellular signaling pathways in species such as human, mouse and bovine (Wang et al. 2004; Adjaye et al. 2005; Chazaud et al. 2006; Xie et al. 2010; Aghajanova et al. 2012). Together, all these pathways form complex signaling machinery, either by acting in parallel or in series, in agonistic or antagonistic manners. Among the several signaling pathways present in early embryos, Notch and Wnt stand out due to their evolutionary well conserved mechanisms that are known to operate synergistically in many adult and embryonic scenarios (Hayward et al.

2008; Muñoz Descalzo and Martinez Arias 2012; Collu et al. 2014). Components and some signaling effects of Notch and Wnt have been identified in early embryos, although their exact role remains elusive and their interplay at this stage of development has not yet been addressed.

2.5.1. Notch signaling pathway

The Notch signaling pathway is an intercellular communication mechanism highly conserved among metazoans, as one or more orthologs of its component proteins can be found from nematodes to mammals (reviewed by Lai 2004; and Kopan and Ilagan 2009). Depending on the context of tissue, specific cellular types at play, cell cycle phase and developmental stage, Notch signaling can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs (Kopan and Ilagan 2009).

In mammals, Notch signaling pathway is composed of four different receptors – Notch 1 to 4 and five canonical ligands – three of the Delta-type (Delta-like – DLL -1, -3 and -4) and two of the serrated-type (Jagged1 and 2) (reviewed by D'Souza et al. 2010). Canonical signaling (Figure 1) is conveyed through the physical interaction of a ligand expressed on the surface of the signaling sending cell, with a receptor expressed on the surface of the signal receiving cell. This interaction leads to the cleavage of the receptor by ADAM-family metalloproteases followed by the final cleavage by an enzyme complex called γ-secretase that contains presenilin, nicastrin, presenilin enhancer 2 (Pen2) and anterior pharynx-defective 1 (Aph1), (reviewed by Bray 2006). This final cleavage releases the Notch intracellular domain (NICD), which is then translocated to the nucleus where it interacts with the DNA-binding protein RBPJ (CSL-protein family). This interaction allows transcriptional co-repressors that were associated to RBPJ to dissociate and transcriptional co-activator Mastermind (Maml) to recognize the NICD/RBPJ interface. The newly formed protein complex then recruits additional co-activators to activate transcription of Notch effector genes, such as *Hes*, *Esr* and *Hey* (reviewed by Kopan and Ilagan 2009; and Borggrefe and Oswald 2009).

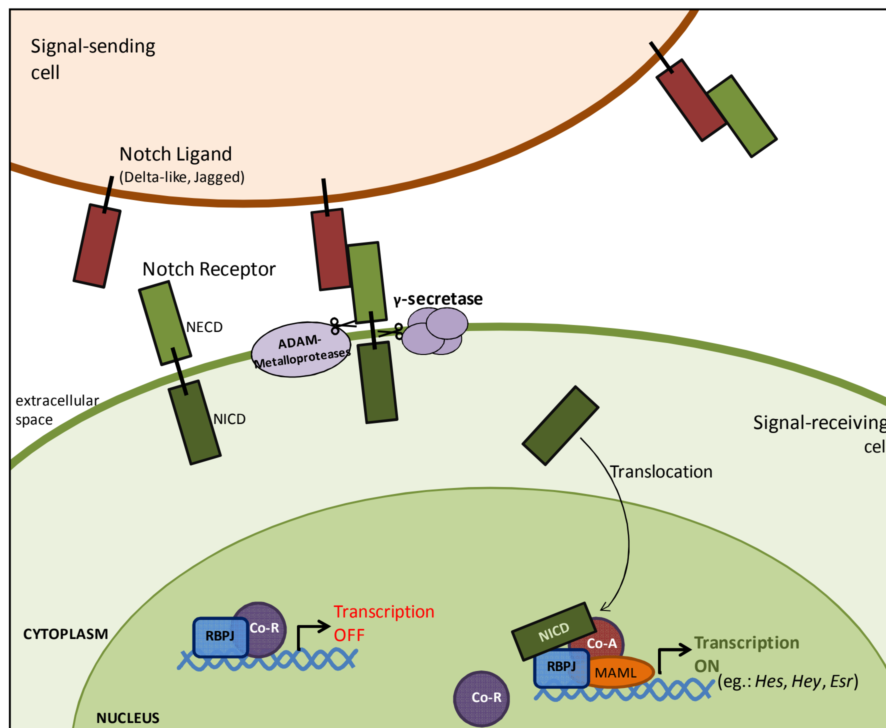

Figure 1: Schematic representation of the core canonical Notch signaling.

The interaction of a ligand of the signal sending cell with the Notch extracellular domain (NECD) of a receptor in the signal receiving cell leads to the enzymatic cleavage of the receptor by ADAM-metalloproteases and the γ-secretase complex, resulting in the release of the Notch intracellular domain (NICD) to the cytoplasm and its translocation to the nucleus. DNA-binding protein RBPJ normally interacts with transcriptional co-repressors (Co-R) to repress transcription. Nuclear NICD interacts with RBPJ recruiting transcriptional co-activator Mastermind (MAML) and other co-activators (Co-A) to activate transcription of Notch effector genes, such as *Hes*, *Esr* and *Hey*.

Modulation of Notch signaling is imperative, given the many possible outcomes of Notch signaling activation in specific biological contexts. Although, theoretically, the many possible ligand-receptor combinations could account for different signaling results, they are not enough to explain the observed differences in signaling activity (Kopanand Ilagan 2009). Since the pathway components undergo proteolysis after signal activation and there is no signal amplification (*i.e.* one ligand activates one receptor that interacts with one CSL protein), regulation of ligand and/or receptor availability is fundamental to control signal intensity (Wu and Bresnick 2007; D'Souza et al. 2008). Modulation of Notch signaling can also be accomplished through post-translational modifications of its components. In fact, glycosylation of the EGF repeats of receptors change their relative response to ligands of the Delta- versus serrate-types (Hicks et al. 2000), and phosphorylation, ubiquitylation, hydroxylation and acetylation regulate receptor’s half-life, as well as contribute to signaling diversity (Andersson et al. 2011). There is also growing evidence that the nuclear settings at one given time influence Notch activity: the location of CSL in the nucleus is dynamic and binding to specific target gene promoters varies, and Notch activity is highly sensitive to

chromatin modifications and histone re-arrangements (Bray 2006). Finally, many studies have uncovered many auxiliary proteins that affect signal transduction and modulate Notch signaling. These can be related to a wide range of cellular functions, like intracellular trafficking (Yeh et al. 2001; Itoh et al. 2003), regulators of NICD and CSL interactions (Jeffries et al. 2002; Wu et al. 2002), or specific ADAM proteases that differentially cleave Notch according to signaling context (Bozkulak and Weinmaster 2009).

There are also less characterized non-canonical variants of Notch signaling pathway that can be ligand dependent or independent, may not require cleavage of the receptor, or may not require NICD interaction with RBPJ (reviewed by Andersen et al. 2012).

2.5.2. Notch signaling in early embryo development

Several studies have identified Notch pathway components in mouse, rat and human preimplantation embryos. The first systematic and semi-quantitative analysis of the presence of Notch components and some of its known regulators in mouse preimplantation embryos was performed by Cormier et al. (2004) in pools of embryos. Subsequent studies have also identified one or more Notch related components in embryos of several developmental stages and of specific embryonic cell lineages using microarray techniques (Wang et al. 2004; Adjaye et al. 2005; Aghajanova et al. 2012; Casanova et al. 2012). Although the presence of Notch components at these embryonic stages is well documented, the exact role of Notch signaling is still controversial.

Earlier studies, using genetically mutant knockout mice to abrogate essential components of canonical Notch signaling, showed that it was dispensable for blastocyst formation and preimplantation development. In fact, knock-out embryos for both presenilins, which are essential components of the γ-secretase complex (Donoviel et al. 1999; Herreman et al. 1999); for protein O-fucosyltransferase 1 (Shi et al. 2005) and O-glucosyltransferase Rumi (Fernandez-Valdivia et al. 2011), which are both essential in priming all Notch receptors to interact with Delta- and serrated-type ligands; and RBPJ itself (Oka et al. 1995; Souilhol et al. 2006), only showed a lethal phenotype after implantation and initiation of gastrulation before day 9.5 of gestation.

However, other studies using knockout mouse embryos for the murine ortholog of *Notchless* (Cormier et al. 2006) and for the murine ortholog of *Brainiac* (Vollrath et al. 2001), which are both Notch signaling regulators, demonstrated a phenotype of embryonic death shortly after implantation. In the case of knockout embryos for the ortholog of *Notchless*, it was demonstrated that the observed lethality was due to the increased apoptosis of ICM

cells (Cormier et al. 2006). Similarly, Chu et al. (2011) demonstrated delayed embryo development and decreased implantation ability after embryo *in vitro* culture with γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). Additionally, more recent studies using mutant knockout embryos for RBPJ and TE regulators (Rayon et al. 2014; Watanabe et al. 2017) unveiled a role for the Notch pathway, together with the Hippo pathway, on TE lineage assignment.

2.5.3. Wnt signaling pathway

Similarly to Notch signaling, the Wnt signaling pathway is a highly conserved intercellular communication mechanism that can be found in all metazoans (Cadigan and Liu 2006). However, unlike Notch and other signaling pathways, Wnt is unusually complex in its machinery and is the target of extensive feed-back control. The activity of Wnt signaling activates a series of transcriptional programs, which are responsible for a wide range of cellular regulatory processes and can be used to control cellular proliferation and differentiation, cell polarity and cell adhesion, depending on cell type and context (Nusse 2005). It is known to operate both in embryogenesis to influence the fate or behavior of neighboring cells, and in adult tissues by maintaining the pool of stem cells necessary for tissue homeostasis (Cadigan and Liu 2006).

Wnt signaling can be divided into three major pathways for signal transduction. The most studied and better understood is the canonical signaling, also called Wnt/β-catenin dependent pathway (Figure 2). The non-canonical β-catenin independent ways can be further divided into the Planar Cell Polarity (PCP) pathway and the Wnt/Calcium (Ca^{2+}) pathway. In addition to these three major branches, ongoing studies are continuously uncovering other signaling pathways in which Wnt can operate (reviewed by Komiya and Habas 2008).

Signal transduction begins with the activation of a receptor by a Wnt ligand. Wnt ligands are secreted lipid-modified glycoproteins (Willert et al. 2003), which are relatively insoluble and rely on heparan sulfate proteoglycans in the extracellular matrix (reviewed by Lin 2004) for diffusion and consequent signaling modulation in short range distances (Clevers and Nusse 2012). There are 19 Wnt ligands in mammals (reviewed by MacDonald et al. 2009) and, while some preferentially activate the β-catenin dependent pathway (e.g.: Wnt1, Wnt3a and Wnt8) and others the β-catenin independent pathway (e.g.: Wnt5a and Wnt11), studies have shown that canonical activation can be accomplished by non-canonical ligands and vice-versa (reviewed by Cadigan and Liu 2006).

The primary receptors for Wnt ligands are Frizzled (Fz) proteins, which are seven-pass transmembrane receptors, although there are other membrane proteins capable of conveying Wnt signaling, such as Ror2 and Ryk (reviewed by van Amerongen et al. 2008). So far, ten Fz receptors have been identified in mammals and, similarly to Wnt ligands, they can be preferentially used by canonical, non-canonical or both types of signaling pathways (Ueno et al. 2013). The activation of canonical and non-canonical signaling in a given cell is mutually exclusive, since there is a competition at the surface of the cell for Fz receptors (Grumolato et al. 2010).

To convey canonical signaling, Fz receptors also require the presence of low density lipoprotein receptor-related proteins 5/6 (LRP5/6) which function as co-receptors. In the event of canonical Wnt activation, Fz and LRP5/6 form a complex at the cellular membrane that leads to the phosphorylation of the cytoplasmic phosphoprotein Dishevelled (Dvl) (Komiya and Habas 2008). The phosphorylation of Dvl can occur with other types of Wnt signaling transduction and it is at this level that Wnt signaling can branch into its aforementioned three major signaling pathways: the stabilization of β-catenin (canonical pathway), the regulation of the cellular cytoskeleton independently of transcription (PCP pathway), and the intracellular Ca^{2+} release from the endoplasmic reticulum (Wnt/Ca^{2+} pathway) (Komiya and Habas 2008). It is also due to Dvl involvement that Wnt can crosstalk with other signaling pathways, namely Notch, as will be described bellow (Muñoz-Descalzo et al. 2010; Collu et al. 2012). The regulation of Dvl functions depends on cellular context, its post-translational modifications, stability and cellular localization (Kim et al.2013).

The central player in canonical Wnt signaling is β-catenin. This molecule has an important dual role in the cell: as a component of cadherin-mediated adherens junctions, and as a key transcriptional co-activator of Wnt signaling (Rudloff and Kemler 2012). In the absence of Wnt signal transduction, the cytoplasmic pool of β-catenin (which is not engaged in cellular adhesion functions) is continually phosphorylated and targeted for proteasomal degradation by the β-catenin destruction complex. This complex is a dynamic multiprotein assembly which core components include glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1), protein phosphatase 2A (PP2A), the scaffolding protein Axin, adenomatosis polyposis coli (APC) protein and the E3-ubiquitin ligase β-TrCP (Stamos and Weis 2013). In the event of Wnt activation, a complex of Wnt-Fz-LRP6 is thought to be formed at the cellular membrane (MacDonald et al. 2009), which in turn recruits Axin and the associated GSK3β to phosphorylate the intracellular domain of LRP. This relocation of the destruction complex results in the inhibition of ubiquitination and proteasomal targeting of the phosphorylated β-catenin (Li et al. 2012), thus saturating the existing β-catenin destruction complexes. Newly

synthesized β-catenin is therefore stabilized by not being affected by phosphorylation and ubiquitination (Staal et al. 2002).

The accumulation of non-phosphorylated β-catenin in the cytoplasm leads to its translocation to the nucleus (Wu et al. 2008; Zhang et al. 2011), where it forms a complex with the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. In the absence of Wnt signaling, TCF/LEF act as transcriptional co-repressors by binding to members of the Groucho/TLE family. The interaction of β-catenin with TCF/LEF leads to the displacement of Groucho/TLE, converting the TCF/LEF repressor complex into a transcriptional activator complex which engages Wnt target gene expression (Daniels and Weis 2005). Mammals have four TCF/LEF genes (TCF1, LEF1, TCF3 and TCF4) and their post-transcriptional modifications and differential promoter usage allow for a wide range of TCF variants with distinct properties and consequent Wnt signaling outcomes (reviewed by Hoppler and Kavanagh 2007).

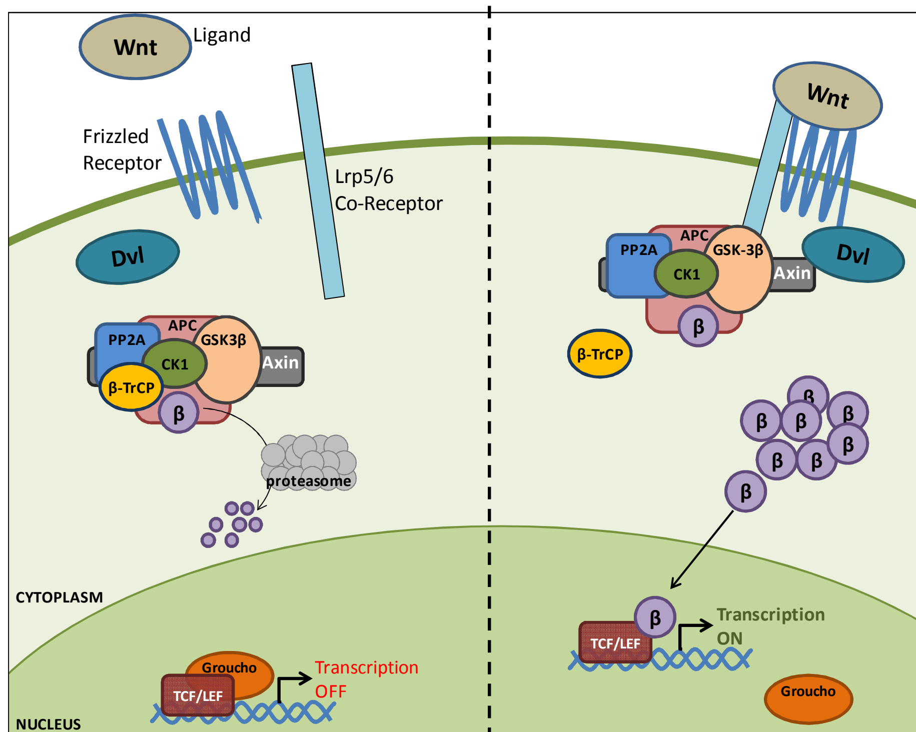

Figure 2: Schematic representation of the canonical (β-catenin dependent) Wnt signaling. Schematic representation of the canonical (β-catenin dependent) Wnt signaling. In the absence of Wnt signaling (on the left), the β-catenin destruction complex (composed of proteins Axin, GSK3β, CK1, PP2A, APC and β-TrPC) leads to the proteosomal degradation of cytoplasmic β-catenin (β). In the case of Wnt signaling activation (on the right), the interaction of a Wnt ligand with a Frizzled receptor and LRP5/6 co-receptors leads to the phosphorylation of Disheveled (Dvl) and interaction with the β-catenin destruction complex. This interaction with the β-catenin destruction complex leads to the inactivation of the final step of the β-catenin destruction process and newly accumulated β-catenin translocates to the nucleus where it binds to TCF/LEF proteins to displace Groucho and activate transcription of Wnt target genes.

In addition to the many possible combinations of ligands, receptors, co-receptors and nuclear effectors which fine-tune Wnt signaling, there are also a wide variety of secreted and transmembrane Wnt antagonists. Among these, the best characterized is the Dickkopf (DKK) protein family, which in vertebrates comprises four members (DKK1-4) (Cruciat and Niehrs 2013). These are evolutionarily well conserved secreted glycoproteins that function as Wnt/β-catenin signaling inhibitors, except DKK3 which regulates the transforming growth factor-β (TGF-β) signaling (Pinho and Niehrs 2007). DKK1 is particularly relevant in reproduction and in embryogenesis, since it is involved in uterine function (Li et al. 2008) and in the coordination of migration processes during pregastrulation (Kimura-Yoshida et al. 2005). It acts by binding to LRP6 with high affinity, competing with Wnt ligands and disrupting the Wnt-induced Fz-LRP6 complex formation, although its effect on promoting LRP6 endocytosis remains controversial (Semënov et al. 2008; Li et al. 2010).

2.5.4. Wnt signaling in early embryo development

The first description of Wnt transcripts in early embryos was done by Lloyd et al. (2003) who identified *Wnt3a* and *Wnt4* in mouse morulae and blastocysts. Subsequent studies further identified a dynamic transcription of more Wnt signaling pathway components throughout mouse early embryonic development (Mohamed et al. 2004a; Mohamed et al. 2004b; Hamatani et al. 2004; Wang et al. 2004; Kemp et al. 2005; Na et al. 2007) hinting at a functional role for this signaling pathway at this stage. However, the exact role of Wnt signaling at these early stages has remained unclear, as conflicting results have been published by several authors.

The main focus of research has been the β-catenin dependent Wnt signaling and efforts have been made to find evidence of its activity in the early embryo. Although work done on mouse and human embryonic stem cells has shown that activation of the canonical Wnt signaling is necessary for maintaining their pluripotent state (Sato et al. 2004), identification of active intra-nuclear β-catenin renders contradicting results, as it has been found in the ICM of mouse blastocysts (Wang et al. 2004), exclusively in the TE of blastocysts (Xie et al. 2008), or not present at any stage (Mohamed et al. 2004b; Na et al. 2007). The manipulation of β-catenin dependent Wnt signaling has also provided divergent results. While many studies found evidences that canonical Wnt activity is essential in post implantation processes such as correctly timed epithelial-mesenchymal transition of the embryonic ectoderm (Kemler et al. 2004) and specification of cells to form the primitive streak and node (Mohamed et al. 2004b), they found no detectable phenotype during preimplantation development.

More recent work has uncovered evidences for a putative role of canonical Wnt signaling in preimplantation embryo development, in the regulation of the first cell fate specification and in embryonic ability to hatch and implant, but with dissonant results across species. Wnt blockade using DKK1 decreased embryo implantation in mice (Xie et al. 2008) while it increased pregnancy rates in bovine (Denicol et al. 2014). At the cellular differentiation and proliferation level, these studies also provided evidence for the involvement of canonical Wnt signaling in the regulation of TE fate specification. In fact, DKK1 treatment increased total cell number in pig blastocysts (Lim et al. 2013) while decreasing it in bovine blastocysts (Denicol et al. 2014), although decreasing the ICM:TE ratio in both species. Conversely, canonical Wnt activation using different strategies also impaired embryo implantation in mice (Li et al. 2005) and decreased embryo total cell number and TE committed cells in bovine and pig embryos (Denicol et al. 2013; Lim et al. 2013).

2.5.5. Notch-Wnt crosstalk

Notch and Wnt are both evolutionarily well conserved intercellular signaling pathways that, together with a wider set of intercellular signaling pathways, operate as a part of a complex mechanism that regulates cellular events such as proliferation and differentiation, and are essential in maintaining tissue functions and homeostasis. Their interplay has been observed in many biological scenarios of vertebrates like the skin wound healing process (Shi 2015), angiogenesis (Yamamizu et al. 2010) and small intestine tissue homeostasis (Tian et al. 2015) in adulthood; in embryogenesis, during somitogenesis (Dequéant et al. 2006; Rallis et al. 2010; Wang et al. 2013), central nervous system development (Cheng et al. 2004) and vascular remodeling and specification (Corada et al. 2010); and also in neoplasic phenomena which may arrive from the deregulation of this interplay as observed in breast cancer (Ayyanan et al. 2006), colon rectal cancer (Ungerbäck et al. 2011) and ovarian carcinoma (Chen et al. 2010), among others.

Signaling pathways, such as Notch and Wnt, are known to interplay in one or more of the following main molecular mechanisms: co-operative regulation of transcriptional targets, transcriptional targets of one pathway affecting the other, and direct molecular crosstalk between the signal transduction machinery (Collu et al. 2014). These three molecular mechanisms are briefly described below and a few examples are provided in Table 1.

Table 1: Examples of Notch and Wnt interplay mechanisms.

Interplay mechanism	Brief description	Biological scenario	Reference
Co-operative regulation: activation of both signaling pathways to allow a specific cellular event	• A complex formed by NICD, β-catenin and RBPJ activates the transcription of specific genes – both the cleavage of a Notch receptor and the stabilization of β-catenin are required.	Vascular progenitor cells (mouse)	Yamamizu et al. (2010)
		Neural precursor cells (mouse)	Shimizu et al. (2008)
Transcriptional targets of one pathway affecting the other: one pathway directly regulates the level of the other's activity	• The activation of LEF1 regulates the expression of DLL1.	Somitogenesis (mouse)	Galceran et al. (2004)
	• The stabilization of β-catenin upregulates *Dll4* transcription, increasing Notch signaling.	Embryonic endothelium (mouse)	Corada et al. (2010)
	• JAGGED1 upregulation increases WNT1 and β-catenin protein expression.	Skin in wound healing processes (rats)	Shi et al. (2015)
	• Wnt activation increases JAGGED1 and HES1 expression • Notch inhibition decreases WNT3a and β-catenin expression.	Dental pulp stem cells (human)	Uribe-Etxebarria et al. 2017
Direct molecular crosstalk: pathways share components of each other's machinery	• Dvl interacts with nuclear CSL, decreasing the availability of its active form, downregulating Notch signaling.	Embryonic epidermis (*Xenopus* frog)	Collu et al. (2012)
	• Dvl binds to NICD affecting its internalization and nuclear trafficking.	0–16 hour old *Drosophila* (fly) embryos	Muñoz-Descalzo et al. (2010)
	• GSK3β phosphorylates the NICD domain of Notch receptors, altering their stability and signaling transduction ability	Embryonic fibroblasts (mouse)	Foltz et al. (2002)
		HEK-293T cells	Espinosa et al. (2003)
	• Membrane bound Notch associates with non-phosphorylated β-catenin, reducing its availability for interactions with nuclear TCF/LEF	Cardiac progenitor cells (mouse)	Kwon et al. (2009)
		Embryonic stem cells (mouse and human)	Kwon et al. (2011)
	• Notch signaling leads to histone modifications at Wnt target gene promoters, disabling their activation	Colorectal cancer cells (human)	Kim et al. (2012)
	• Wnt/β-catenin signaling directly activates a HES1 promoter (independently of Notch activation)	Colorectal adenocarcinoma cells (mouse and human)	Peignon et al. (2011)
	• NICD stimulates LEF1 activity (independently of other Wnt components)	NIH3T3 cells (mouse); Jurkat cells (human)	Ross and Kadesch (2001)

The diversity of possible Notch-Wnt interactions is immense and it is important to understand that the existence of these interactions and their nature are cell and context specific (Andersson et al. 2011). For example, while the NICD, β-catenin and RBPJ complex is formed in vascular progenitors and neural precursor cells (Shimizu et al. 2008; Yamamizu et al. 2010), it is not found in venous endothelial cells (Yamamizu et al. 2010). Therefore, it is important to study each biological scenario individually and understand which, if any, of the cross-talk mechanisms are in play.

2.5.6. Notch and Wnt signaling in cellular differentiation and pluripotency maintenance

As previously described, Notch and Wnt signaling pathways have frequently been associated with cell fate decisions and pluripotency maintenance. As such, Notch and Wnt have been implicated as regulators or as regulation targets of transcription factors associated with pluripotency maintenance – SOX2, OCT4 and KLF4, and differentiation associated transcription factor CDX2 in embryogenesis and adulthood. The mechanisms through which these pathways operate is still being resolved, although there seems to be no conserved mechanism by which this cross-regulation is achieved, as they vary according to cell type and tissue context in the different biological scenarios that have been addressed so far.

Since these transcription factors are involved in so many cellular regulatory processes it is easy to understand that they act, not only downstream, but also upstream of several signaling pathways in order to finely tune their own availability in a given cell and tissue, as well as the intensity of the involved signal transduction pathways.

Notch signaling is known to be involved, both in its canonical and non-canonical variants, in the regulation of SOX2, OCT4, KLF4 and CDX2 in several cellular contexts, and also as the target of these transcription factors. A few examples will be provided below in Table 2.

Table 2: Examples of Notch signaling interaction with pluripotency markers SOX2, KLF4 and OCT4 and differentiation marker CDX2.

Interaction	Brief description	Biological scenario	Reference
Notch vs.SOX2	• Ligand JAGGED1 maintains SOX2 expression within restricted domains of the otic epithelium	Embryonic inner ear (chicken)	Neves et al. (2011)
	• Activation of receptor NOTCH2 with JAGGED1 ligand, involving HES1 activation maintains SOX2 expression	Embryonic and adult anterior pituitary gland (rat)	Batchuluun et al. (2017)
	• Suppression of NOTCH1 increases SOX2 expression • Suppression of NOTCH2, NOTCH3 and NOTCH4 decreases SOX2 expression	Colon cancer stem cells (human)	Apostolou et al. (2013)
	• SOX2 regulates NOTCH1 transcription and expression	Retinal neural progenitor cells (mouse)	Taranova et al. (2006)
Notch vs. KLF4	• NICD binds to *Klf4* target promoter and represses its transcription	Small intestine and colon mucosa cells (mouse)	Zheng et al. (2009)
		Embryonic fibroblasts and other cell lines (mouse)	Han et al. (2017)
		Endothelium (mouse)	Hale et al. (2014)
	• KLF4 induces the transcription of *Notch1*, *Dll1*, *Dll4* and *Hey2*	Endothelium (mouse)	Hale et al. (2014)
Notch vs. OCT4	• Notch activation induces OCT4 expression	Dental pulp stem cells (human)	Uribe-Etxebarria et al. (2017)
	• OCT4 induces *Notch1*, *Notch2* and *Hes1* transcription	*In vitro* cultured hepatocytes(rat)	Doffou et al. (2018)
	• Suppression of NOTCH1, NOTCH3 and NOTCH4 increases OCT4 expression • Suppression of NOTCH2 decreases OCT4 expression	Colon cancer stem cells (human)	Apostolou et al. (2013)
Notch vs. CDX2	• Notch (in association with Hippo) signaling activation induces CDX2 expression	Blastocyst (but not at post implantation stages) (mouse)	Rayon et al. (2014); Rayon et al. (2016)
	• DLL1 expression, through non-canonical mechanisms, increases *Cdx2* transcription and expression • CDX2 increases DLL1 transcription and expression	Esophageal epithelial cells (human)	Tamagawa et al. (2016)
	• Canonical Notch blockade increases CDX2 expression • CDX2 expression decreases *Hes1* transcription and expression	Esophageal epithelial cells (human)	Tamagawa et al. (2012)
	• CDX2 occupies *Dll1* promoter increasing its transcription	Embryonic intestinal tracts (mouse)	Grainger et al. (2012)
	• CDX2 overexpression upregulates *Notch1* and *Notch3* transcription	Intestinal epithelial cells (mouse)	Uesaka et al. (2004)

The complexity of Wnt signaling allows many of its components to be responsible for the regulation of the aforementioned pluripotency/ differentiation associated transcription factors. Similarly to what we observe in Notch signaling, Wnt can also be regulated by these transcription factors. For instance, Sox proteins can regulate canonical Wnt signaling by a number of different mechanisms (Kormish et al. 2010). Examples of cross regulation are listed below in Table 3.

Table 3: Examples of Wnt signaling interaction with pluripotency markers SOX2, KLF4 and OCT4 and differentiation marker CDX2.

Interaction	Brief description	Biological scenario	Reference
Wnt vs. SOX2	• Downregulation of canonical Wnt (downregulation of *Tcf1* and *Lef1* transcription) allows SOX2 expression	Bronchiolar development (mouse)	Hashimoto et al. (2012)
	• Knockdown of SOX2 inhibits canonical Wnt signaling (downregulation of GSK3β and β-catenin expression)	*In vitro* osteosarcoma cells (human)	Tang et al. (2018)
	• Wnt signaling is reduced by SOX2 sequestring of β-catenin in the nucleus	Osteoblasts (mouse)	Mansukhani et al. (2005)
Wnt vs. OCT4	• Canonical Wnt signaling upregulates OCT4 expression	Embryonic fibroblasts (mouse)	Zhang et al. (2014)
	• Wnt signaling is reduced by OCT4 sequestring of β-catenin in the nucleus and inducing its phosphorilation	ES cells (mouse)	Abu-Remaileh et al. (2010)
	• TCF3 inhibits *Oct4* transcription by targeting its promoter	ES cells (mouse)	Kelly et al. (2011); Tam et al. (2008)
	• OCT4 forms a complex with β-catenin to activate OCT4 targets (independently of TCF/LEF-mediated transcription)	ES cells (mouse)	Kelly et al. (2011)

Table 3 (continuation)

Interaction	Brief description	Biological scenario	Reference
Wnt vs. KLF4	• Canonical Wnt activation allows β-catenin to bind to KLF4 promoter, inducing its transcription and expression	ES cells and induced pluripotent stem cells (mouse)	Zhang et al. (2014); Ai et al. (2016)
	• APC is required for *Klf4* transcription and expression	Colonic epithelial cells (human)	Stone et al. (2002)
	• KLF4 controls β-catenin transcription	Colonic epithelial cells (human)	Stone et al. (2002)
		Corneal epithelial cells (mouse)	Tiwari et al. (2017)
	• Wnt signaling is reduced by KLF4 sequestring of β-catenin in the nucleus	*In vitro* cultured lung and colonic cancer cells (human)	Sellak et al. (2012)
		Intestinal epithelial cells (human)	Zhang et al. (2006)
	• KLF4 controls WNT5a (non-canonical) transcription and expression	Keratinocytes of stratified epithelia (mouse)	Tetreault et al. (2016)
Wnt vs. CDX2	• Wnt activation induces CDX2 expression • CDX2 influences β-catenin and its destruction complex component levels and controls Wnt signal transduction	Intestinal epithelial cells (pig)	Fan et al. (2017)
	• β-catenin-TCF complexes activate *Cdx2* transcription	Embryonic caudal neural tube (mouse)	Zhao et al. (2014)
	• CDX2 increases *Wnt3a* transcription and expression	Paraxial mesoderm (mouse)	Savory et al. (2009)
	• Wnt signaling is reduced by CDX2 sequestring of β-catenin in the nucleus	Colon cancer cells (human)	Guo et al. (2010)
		Endometrial carcinoma (human)	Saegusa et al. (2007)
		Lung cancer cell lines (human)	Liu et al. (2012)

3. EXPERIMENTAL WORK

3.1. Chapter I – Notch signaling in mouse blastocyst development and hatching

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3.1.1. Abstract

Background: Mammalian early embryo development requires a well-orchestrated interplay of cell signaling pathways. Notch is a major regulatory pathway involved in cell-fate determination in embryonic and adult scenarios. However, the role of Notch in embryonic pre-implantation development is controversial. In particular, Notch role on blastocyst development and hatching remains elusive, and a complete picture of the transcription and expression patterns of Notch components during this time-period is not available.

Results: This study provided a comprehensive view on the dynamics of individual embryo gene transcription and protein expression patterns of Notch components (receptors Notch1-4; ligands Dll1 and Dll4, Jagged1-2; and effectors Hes1-2), and their relationship with transcription of gene markers of pluripotency and differentiation (*Sox2*, *Oct4*, *Klf4*, *Cdx2*) during mouse blastocyst development and hatching. Transcription of *Notch1-2*, *Jagged1-2* and *Hes1* was highly prevalent and dynamic along stages of development, whereas transcription of *Notch3-4*, *Dll4* and *Hes2* had a low prevalence among embryos. Transcription levels of *Notch1*, *Notch2*, *Jagged2* and *Hes1* correlated with each other and with those of pluripotency and differentiation genes. Gene transcription was associated to protein expression, except for Jagged2, where high transcription levels in all embryos were not translated into protein. Presence of Notch signaling activity was confirmed through nuclear NICD and HES1 detection, and downregulation of *Hes1* transcription following canonical signaling blockade with DAPT. *In vitro* embryo culture supplementation with JAGGED1 had no effect on embryo developmental kinetics. In contrast, supplementation with JAGGED2 abolished *Jagged1* transcription, downregulated *Cdx2* transcription and inhibited blastocyst hatching. Notch signaling blockade by DAPT downregulated transcription of *Sox2* and retarded embryo hatching.

* Text adapted from the original published paper

Conclusion: Transcription of Notch genes showed a dynamic pattern along blastocyst development and hatching. Data confirmed Notch signaling activity, and lead to the suggestion that Notch canonical signaling may be operating through Notch1, Notch3, Jagged1 and Hes1. Embryo culture supplementation with JAGGED1 and JAGGED2 unveiled a possible regulatory effect between Jagged1, CDX2 and blastocyst hatching. Overall, results indicate that a deregulation in Notch signaling, either by its over or under-activation, affects blastocyst development and hatching.

Keywords: Blastocyst; development; hatching; Notch; mouse

3.1.2. Introduction

Abnormal mammalian preimplantation embryo development is responsible for a significant prevalence of embryo-fetal mortality in both human and domestic animal species (Diskin and Morris 2008; Jarvis 2016). However, the complex spatial and temporal orchestration of cellular events associated with early development, which require a finely tuned inter-cellular communication, is still largely unresolved. Zygote cleavage leads to the compact morula stage, where the first cellular differentiation events originate the blastocyst (Rossant and Tam 2009). The blastocyst comprises two cell types: i) trophectoderm (TE) – which will give rise to the placenta, and ii) inner-cell-mass (ICM) – which will constitute the embryo itself (Marikawa and Alarcon 2012). The maintenance of TE epithelial integrity and differentiated status relies on transcription factor CDX2 expression (Strumpf et al. 2005; Ralston and Rossant 2008). Likewise, ICM pluripotency maintenance relies on expression of a wide network of transcription factors, namely SOX2, OCT4 and KLF4 (Wei et al. 2009, Schrode et al. 2013).

Several cell signaling pathways critical for embryo development have been identified in the mouse preimplantation embryo (Zhang et al. 2007). The Notch cell signaling pathway, highly conserved among invertebrates and vertebrates, has been implicated as a main regulator of cellular differentiation and proliferation in many adult and embryonic scenarios (Bray 2006; Meier-Stiegen et al. 2010; Andersson et al. 2011; Watanabe et al. 2017), and was identified in several mammalian preimplantation embryos, including the mouse (Cormier et al. 2004; Wang et al. 2004; Aghajanova et al. 2012; Hosseini et al. 2015; Tang et al. 2011; Deng et al. 2014). In mammals, Notch is a receptor-ligand based cell signaling pathway composed of four receptors (Notch1-4) and five ligands (Delta-like (DLL) 1, 3 and 4; Jagged1 and 2). Notch signaling may be conveyed in the so-called canonical and non-canonical

forms, reflecting a high mechanistic complexity, yet to be fully understood (for reviews see Guruharsha et al. 2012; Bray 2016; Alfred and Vaccari 2018). Briefly, the canonical signaling results from the interaction of a ligand expressed by the signal-sending cell with a transmembrane receptor expressed by a signal-receiving neighboring cell. This binding of the ligand in *trans* leads to the sequential cleavage of the intracellular domain (NICD) of the receptor by extracellular ADAM proteases and an intracellular γ-secretase, and its translocation to the nucleus. Here, NICD de-represses the transcription complex RBPJ, to regulate the transcription of Notch effector genes (including *Hes1* and *Hes2*). Signal termination is ensured by ubiquitin-dependent proteasome degradation of NICD. Knowledge on non-canonical Notch signaling in mammalian systems is still largely fragmentary (Alfred and Vaccari, 2018). This form is ligand independent and/or does not require NICD interaction with RBPJ (Andersen et al. 2012). The role of Notch signaling in embryo preimplantation development is controversial. Earlier studies reported that canonical Notch signaling is not required for early embryo development (Shi et al. 2005; Souilhol et al. 2006), although subsequent studies showed that pharmacological inhibition of the pathway with DAPT (a γ-secretase inhibitor) affects embryo implantation (Chu et al. 2011). More recently, studies using mutant knockout embryos unveiled a role for Notch, together with the Hippo pathway, on TE lineage assignment (Rayon et al. 2014; Watanabe et al. 2017; Menchero et al. 2019).

This study considered the evaluation of Notch signaling, in individual embryos, in a defined time-frame of mouse preimplantation embryonic development – blastocyst differentiation from compact morulae until blastocyst hatching. This evaluation included gene transcription (quantitative real-time PCR; qRT-PCR), protein expression (immunocytochemistry; ICC), and *in vitro* embryo culture supplementation with Notch activators and inhibitors. In transcription analysis, the first step was to identify the prevalence of transcription of Notch (receptors, ligands and effectors) and pluripotency and differentiation genes along four developmental stages: compact morulae (CM), blastocyst (BL), expanded blastocyst (EBL) and hatched blastocyst (HBL). The second step was to evaluate the levels of transcription of each gene at each developmental stage. The above data allowed the evaluation of transcription relationships (correlations) between Notch and pluripotency and differentiation genes. Evaluation of protein expression by ICC at the BL stage evidenced mRNA translation and the nuclear identification of NICD and/or effectors, thus confirming Notch signaling activity. Finally, *in vitro* embryo culture with a γ-secretase (Notch signaling blockade) or with Notch ligands (putative activators) evidenced phenotypic effects in embryo development and gene transcription. Therefore, the objectives of this study were, at the individual embryo level, to evaluate i) the signaling status of Notch pathway and the dynamic patterns of transcription and expression of Notch components, from the compact

morulae stage until blastocyst hatching; ii) the relationship between the transcription of Notch components and gene markers of embryonic pluripotency and differentiation; and iii) the effects of supplementation with Notch ligands and Notch signaling inhibitors on blastocyst development and hatching.

3.1.3. Materials and Methods

3.1.3.1. Animals

Animal manipulation and experimental procedures were conducted according to the national and European Union legislation regarding the use of animals for experimental purposes, and under the license of the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal; Ref. 001/2018). Male and female Crl: CD1 (ICR) (CD1) mice (*Mus musculus*) were purchased from Charles River Laboratoire France and maintained at the Faculty of Veterinary Medicine of the University of Lisbon animal house facilities. Mice were maintained in a 12 h light/dark cycle, in corn cob bedded cages and with *ad libitum* access to standard laboratory diet and water. Mouse health was monitored daily.

3.1.3.2. Embryo collection and *in vitro* culture

Embryos were obtained from 2-3 months-old CD1 female mice, following superovulation and mating with CD1 males. Briefly, females were injected intraperitoneally with 10 IU equine chorionic gonadotropin (Intergonan; MSD Animal Health, Portugal) and 46 h later with 10 IU human chorionic gonadotropin (hCG; Chorulon; MSD Animal Health). Females were then housed overnight with a male and the presence of a vaginal plug was checked the following morning (0.5 dpc). At 2.5 dpc, females were euthanized by cervical dislocation under general anesthesia (intraperitoneal injection with 150 mg kg^{-1} ketamine + 10 mg kg^{-1} xylazine) and embryos were collected by oviduct flushing with M2 medium (Sigma-Aldrich, St Louis, MO, USA). Morphologically normal 8 to 16-cell embryos were selected, washed in M2 medium and *in vitro* cultured in groups of 20 in 500 µl of KSOM (Millipore, Specialty Media, Germany) overlaid with 400 µl of mineral oil (EmbryoMax®, Millipore), in 4-well dishes (Nunclon, Nunc, Roskilde, Denmark), at 37 ºC in a 90% N_2 + 5% O_2 + 5% CO_2 humidified atmosphere. Following a 24, 36 and 48 h culture (corresponding to

respectively 3.5 dpc, 4.0 dpc and 4.5 dpc), embryos were classified into the CM, BL, EBL, eHBL (early HBL) and HBL developmental stages, according to Nagy et al. (2003) (Figure 3).

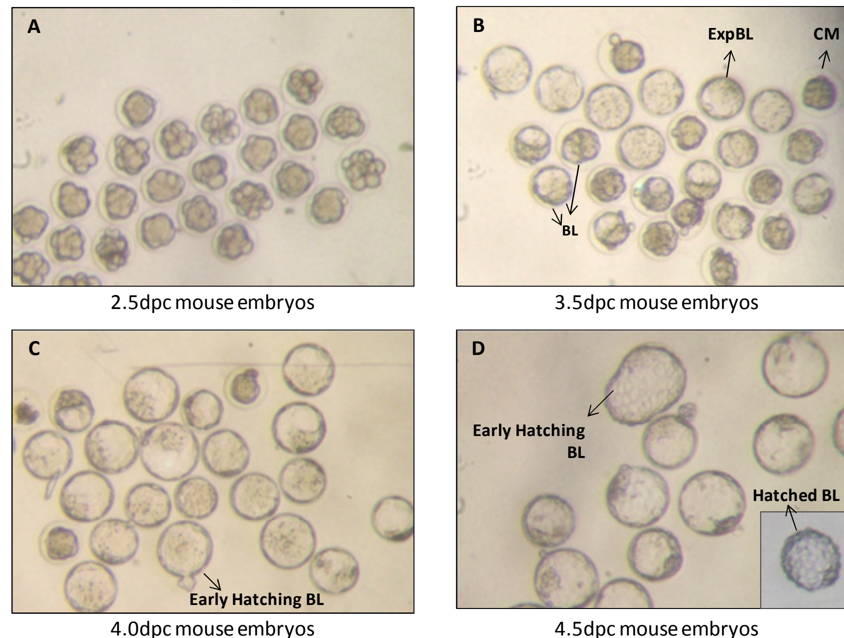


Figure 3: Representative photographs illustrating the morphological staging of embryonic development.

Mouse embryos were *in vivo* collected at 2.5 dpc (A), *in vitro* cultured, and morphologically evaluated according to Nagy et al. (2003) at 3.5 dpc (cultured for 24 h; B), 4.0 dpc (cultured for 36 h; C) and 4.5 dpc (cultured for 48 h; D). CM = compact morula, BL = blastocyst, EBL = expanded blastocysts, eHBL = early hatching blastocyst, HBL = hatched blastocyst.

3.1.3.3. Gene transcription analysis - qRT-PCR

Quantification of transcripts of Notch components – receptors (*Notch1*, *Notch2*, *Notch3* and *Notch4*), ligands (*Delta-like1 - Dll1*, *Delta-like4 - Dll4*, *Jagged1* and *Jagged2*), and effectors (*Hes1* and *Hes2*) – and of transcripts of pluripotency and differentiation gene markers *Sox2*, *Klf4*, *Oct4*, *Cdx2*, *Cdca7* and *Lgr5* was analyzed in individual 3.5 dpc CM (n= 9), BL (n= 9) and EBL (n= 7) and 4.5 dpc HBL (n= 5). Overall, transcription was individually evaluated in 30 embryos.

RNA extraction of single embryos was performed using the Arcturus® PicoPure™ RNA Isolation Kit (Applied Biosystems, ThermoFisher Scientific, USA) and DNA digestion with RNase-free DNase Set (Qiagen, Hilden, Germany). Concentration and purity of RNA were assessed spectrophotometrically at 260 and 280 nm (NanoDrop®2000c, ThermoFisher Scientific). Complimentary DNA (cDNA) synthesis was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific) using 20 ng of total RNA in each

reaction. Pre-amplification of cDNA was achieved with SSoAdvanced™ PreAmp Supermix (BioRad, CA, USA) using 10 µL of undiluted cDNA and a primer pool of genes *Notch1-4*, *Dll1* and *Dll4*, *Jagged1-2*, *Hes1-2*, *Sox2*, *Klf4*, *Oct4*, *Cdx2*, *Lgr5*, and reference genes *Rps29* and *Hprt1* (Annex I). With the exception of *Sox2*, which is coded by a single exon, primers were designed to bracket two exons to avoid genomic DNA amplification. In the case of *Sox2*, the cDNA specific amplification was confirmed with a minus-reverse transcriptase control.

Pre-amplified cDNA was diluted 1:10 in Tris-EDTA buffer and kept at -20 ºC until qRT-PCR analysis. This was performed in duplicate wells in StepOne Plus™ (Applied Biosystems, ThermoFisher Scientific) in 96-well optical reaction plates (Applied Biosystems), using the universal temperature cycles: 10 min of pre-incubation at 95 ºC, followed by 40 two-temperature cycles (15 s at 95 ºC and 1 min at 60 ºC). Melting curves were acquired to ensure that a single product was amplified in the reaction. Each reaction used 10 µL of Perfecta® Sybr® Green Fast Mix, ROX™ (Quanta bio, MA, USA), 2 µL of diluted pre-amplified cDNA (corresponding to 0.2 ng of cDNA) and 80 nM of each primer in a total reaction volume of 20 µL. A NTC (no-template control) was included in all reaction plates and only plates with undetermined Ct in NTC wells were analyzed. Also, only wells with a single specific melting curve peak were analyzed. For each gene, one PCR product was run through a 2.5% agarose gel to confirm expected product size and the identity of this PCR product was confirmed by DNA sequencing. All reactions with the same Tm as the confirmed PCR product were considered specific. Positive controls were added to each reaction plate to exclude primer design artifacts: mouse uterus in oestrus for *Notch1*, *Dll4* and *Hes1* transcription, mouse uterus in metoestrus for *Notch2*, *Notch3* and *Hes2* transcription, mouse uterus in dioestrus for *Notch4*, *Jagged1* and *Jagged2* transcription (Murta et al. 2015), and mouse small intestine for *Dll1* and *Lgr5* (Badenes et al. 2017). Embryos themselves were used as positive controls for *Sox2*, *Klf4*, *Oct4*, *Cdx2* and *Cdca7* transcription (Tang et al. 2011; Deng et al. 2014).

The first step in transcription data analysis was the calculation of prevalence among embryos, i.e. the proportion of embryos with detected transcription at each developmental stage. Genes with a Ct value > 35 were considered without amplification. This was further confirmed by visualization of qRT-PCR products in agarose gels (Table 5 and Figure 4). The next step in transcription analysis was to quantify transcription levels of most prevalent genes. This was performed by two approaches. In Figure 5A, Ct values were normalized to housekeeping gene 1 (*Rps29*) and the ∆Ct values obtained further calibrated with housekeeping gene 2 (*Hprt1*), generating ∆∆Ct values. These values were log transformed and results presented as the Log_2 of power of ∆∆Ct values. The Log_2 of power of ∆Ct values

of transcription levels of housekeeping genes *Rps29* and *Hprt1* at each developmental stage are shown in Figure 5B. The second approach is shown in Figure 5C. Here, Ct values of each target gene were normalized with the mean Ct values of housekeeping genes *Rps29* and *Hprt1*, and the obtained ΔCt values were then calibrated to ΔCt values of compact morulae (shown as 0.0), originating the ΔΔCt values for log transformation (Livak and Schmittgen 2001). Results are also presented as the Log_2 of power of ΔΔCt values.

3.1.3.4. Gene expression analysis - immunocytochemistry

Embryos (3.5 dpc BL) were fixated in a 4% paraformaldehyde solution for 30 min, at 4ºC, permeabilized in phosphate-buffered saline (PBS) + 0.5% Triton X-100 for 1 min and washed in PBS. Blocking was performed in a PBS + 0.1% Tween20 solution containing 2.5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature, followed by a 4 ºC overnight incubation with the primary antibody diluted in blocking solution. Primary antibodies, all polyclonal and already validated for use in mouse cells (Murta et al. 2013; Murta et al. 2015), were diluted as presented in Table 4. Negative IgG controls were performed using rabbit polyclonal IgG (ab27478, Abcam) and goat polyclonal IgG (ab37373, Abcam) at the appropriate dilutions. Embryos were then washed in PBS (4 × 5 min) and incubated with AlexaFluor® 594 chicken anti-rabbit (A11012, Life Technologies, USA) or chicken anti-goat (A21468, Life Technologies) secondary antibody diluted 1:300 in blocking solution, according to primary antibody host species, for 30 min, at room temperature. Embryos were then washed 2 × 10 min in PBS followed by Hoechst33268 (Sigma-Aldrich) nuclear labeling and finally mounted in ProLong™ Gold Antifade Mountant (Life Technologies). For each primary antibody, 6 blastocysts were analyzed, and a Z-stack was captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with an optical magnification of 400× and treated with Fiji software (National Institutes of Health, USA).

Table 4: Primary antibodies used for immunocytochemistry

Antibody	Source	Dilution	Supplier (catalogue number)
anti-NOTCH1	Rabbit polyclonal	1:100	Abcam (ab8925)
anti-NOTCH2	Rabbit polyclonal	1:100	Abcam (ab8926)
anti-NOTCH3	Rabbit polyclonal	1:200	Abcam (ab23426)
anti-NOTCH4	Rabbit polyclonal	1:50	Santacruz Biotechnology (sc5594)
anti-DLL1	Rabbit polyclonal	1:100	Abcam (ab76655)
anti-DLL4	Rabbit polyclonal	1:200	Abcam (ab7280)
anti-JAGGED1	Rabbit polyclonal	1:50	Santacruz Biotechnology (sc8303)
anti-JAGGED2	Goat polyclonal	1:50	Santacruz Biotechnology (sc8158)
anti-HES1	Rabbit polyclonal	1:100	Abcam (ab71559)
anti-HES2	Rabbit polyclonal	1:100	Abcam (ab134685)

Primary antibodies' dilutions and manufacturer and catalogue reference, as used by Murta et al. (2013 and 2015).

3.1.3.5. Embryo culture supplementation with Notch ligands and a Notch signaling inhibitor

Mouse 8-16 cell embryos were collected and *in vitro* cultured as previously described, being randomly allocated in groups of 20 to each of the following treatment groups: i) Control, without treatment ii) JAGGED1, medium supplemented with 1 µg mL^{-1} JAGGED1 (1277-JG, R&D Systems, Bio-Techne, USA); iii) JAGGED2, medium supplemented with 1 µg mL^{-1} JAGGED2 (4748-JG, R&D Systems); and iv) DAPT, medium supplemented with 100 µM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; Sigma-Aldrich). The experiment considered 10 *in vitro* culture sessions (550 embryos) until 3.5 dpc (24 h), from which 9 sessions (511 embryos) were further cultured until 4.0 dpc (36 h), and from the latter, 6 sessions (301 embryos) were further cultured until 4.5 dpc (48 h). Embryos were evaluated for viability, expressed as non-degenerated morphologically normal embryos progressing in culture, and their developmental stage recorded at those time-points (Figure 3) by a technician blinded to group assignment, according to criteria established by Nagy et al. (2003). Five to six individual 4.0 dpc EBL from each group were processed for quantification of transcripts of Notch genes (*Notch1-2*, *Jagged1-2*, *Hes1*) and pluripotency and differentiation marker genes (*Sox2*, *Klf4*, *Oct4*, *Cdx2*, *Cdca7*), as described above.

3.1.3.6. Statistical analysis

Statistical analysis was performed using the statistical software SPSS Statistics (version 22, IBM® SPSS® Statistics, 2013, IBM, NY, USA). Real-time PCR data (ΔCt values) did not follow normal distribution (Annex II) and were transformed to log 2 of power of ΔΔCt for normalization, which allowed the use of parametric tests. Regarding *Notch1*, *Notch2*, *Jagged1*, *Jagged2*, *Hes1*, *Sox2*, *Oct4*, *Klf4* and *Cdx2* transcription, ANOVA was performed to compare the relative transcription between developmental stages, followed by LSD *post-hoc* analysis. Two-sided Pearson correlation coefficient was calculated to investigate the relationship between the transcription of Notch components, and between the latter and the transcription of pluripotency/differentiation markers. Chi-square test was used to evaluate the effect of JAGGED1, JAGGED2 and DAPT medium supplementation on *in vitro* cultured embryo viability and developmental rates. Results were considered significant if $p < 0.05$.

3.1.4. Results

3.1.4.1. Gene transcription

Transcription prevalence and levels of Notch and pluripotency and differentiation genes was analyzed by qRT-PCR in individual embryos at four developmental stages: 3.5 days *post-coitum* (dpc) CM, BL and EBL, and 4.5 dpc HBL. Based on RNA-seq databases (Tang et al. 2011; Deng et al. 2014), *Lgr5* was chosen as negative gene transcription control, as this pluripotency-associated gene showed very low transcription levels in embryonic cells of developmental stages considered in this study. Table 5 shows gene transcription prevalence among individual embryos and stages of development, and Figure 4 illustrates the respective agarose gels of qRT-PCR products (displaying four embryos / gene / stage of development). Regarding Notch genes, transcription of receptors *Notch1* and *Notch2*, ligand *Jagged2* and effector *Hes1* was detected in all embryos, and transcription of ligand *Jagged1* was detected in all but four embryos. Receptors *Notch3* and *Notch4*, ligand *Dll4* and effector *Hes2* had inconsistent transcription among embryos, whereas transcription of ligand *Dll1* was not detected. Transcription of pluripotency and differentiation genes (*Sox2*, *Oct4*, *Klf4*, *Cdx2*) was detected in all embryos, whereas transcription of negative control *Lgr5* was not detected.

Table 5: Prevalence of gene transcription among embryos at each stage of development.

Gene	Stage of development			
	Compact Morula	Blastocyst	Expanded Blastocyst	Hatched Blastocyst
Notch1	9/9	9/9	7/7	5/5
Notch2	9/9	9/9	7/7	5/5
Notch3	5/9	3/9	0/7	2/5
Notch4	0/9	0/9	1/7	2/5
Dll1	0/9	0/9	0/7	0/5
Dll4	0/9	0/9	2/7	1/5
Jagged1	6/9	9/9	7/7	4/5
Jagged2	9/9	9/9	7/7	5/5
Hes1	9/9	9/9	7/7	5/5
Hes2	5/9	3/9	3/7	0/5
Lgr5	0/9	0/9	0/7	0/5
Sox2	9/9	9/9	7/7	5/5
Klf4	9/9	9/9	7/7	5/5
Cdx2	9/9	9/9	7/7	5/5
Oct4	9/9	9/9	7/7	5/5

Prevalence is depicted as the number of embryos with specific amplification of the gene in relation to the total number of embryos analyzed.

Figure 4: Agarose gels representative of the prevalence of gene transcription among embryos at each stage of development.

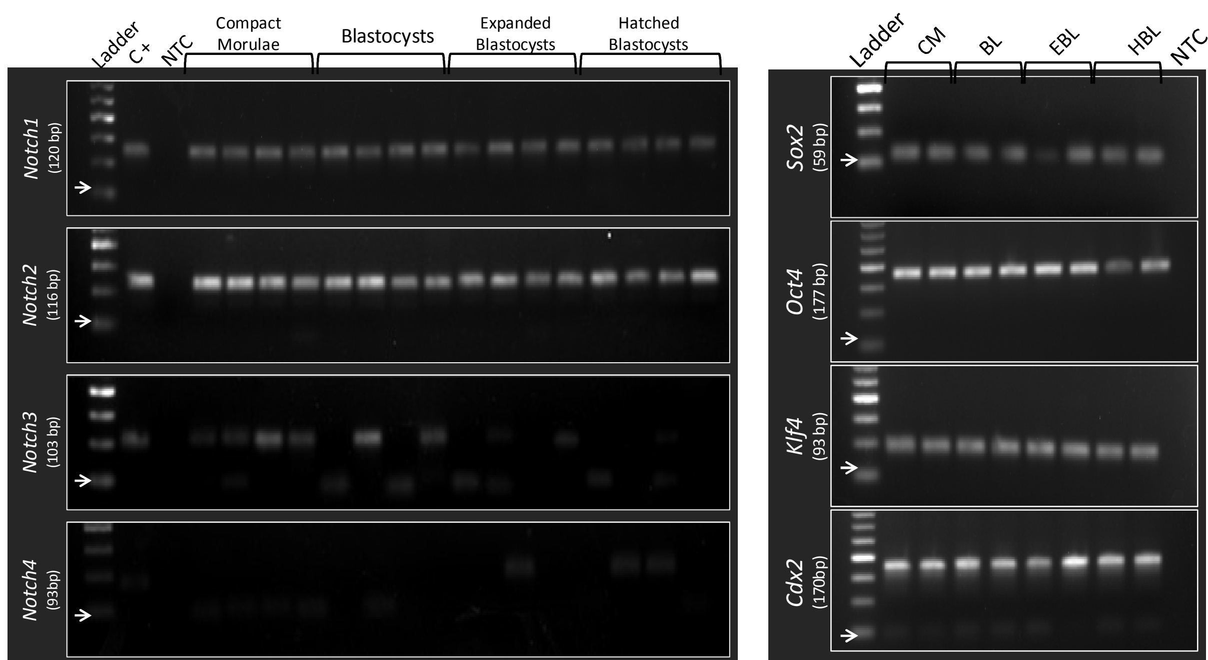
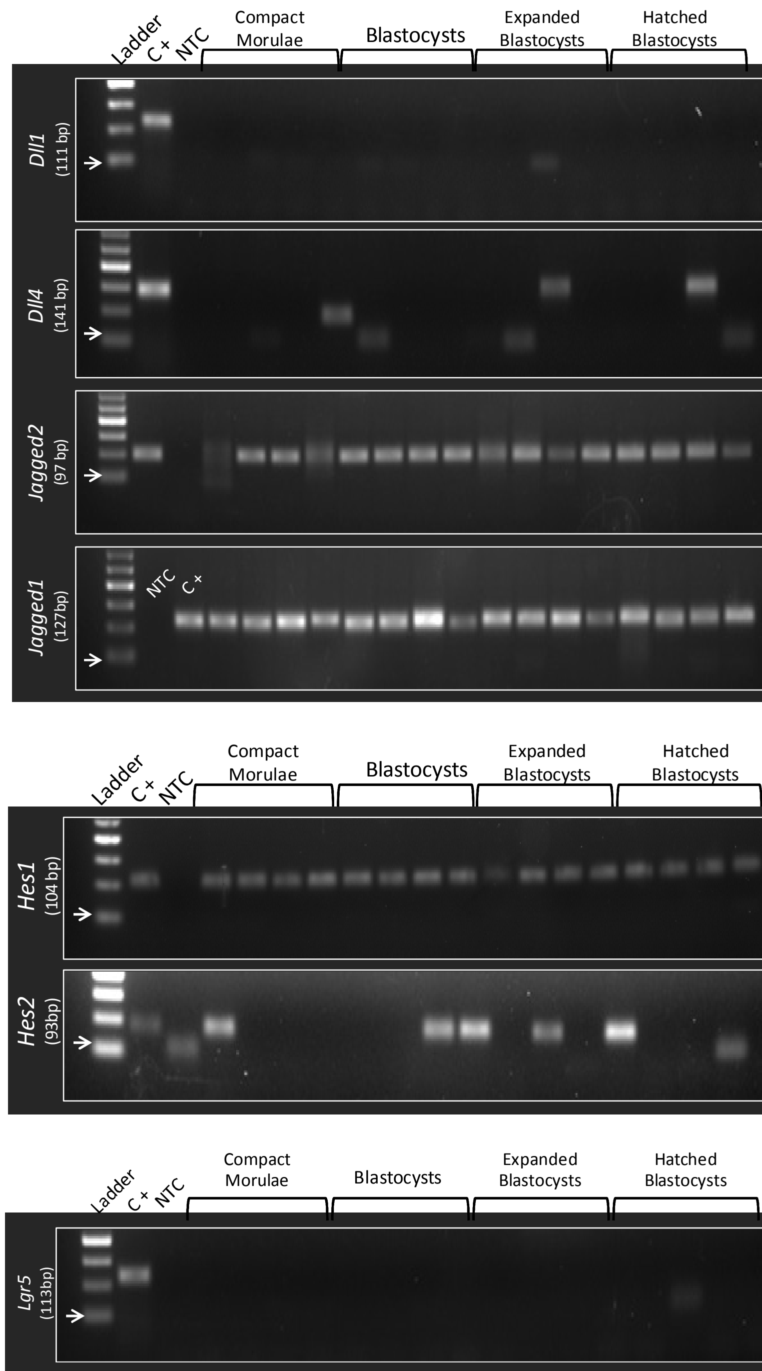


Figure 4 (continuation)

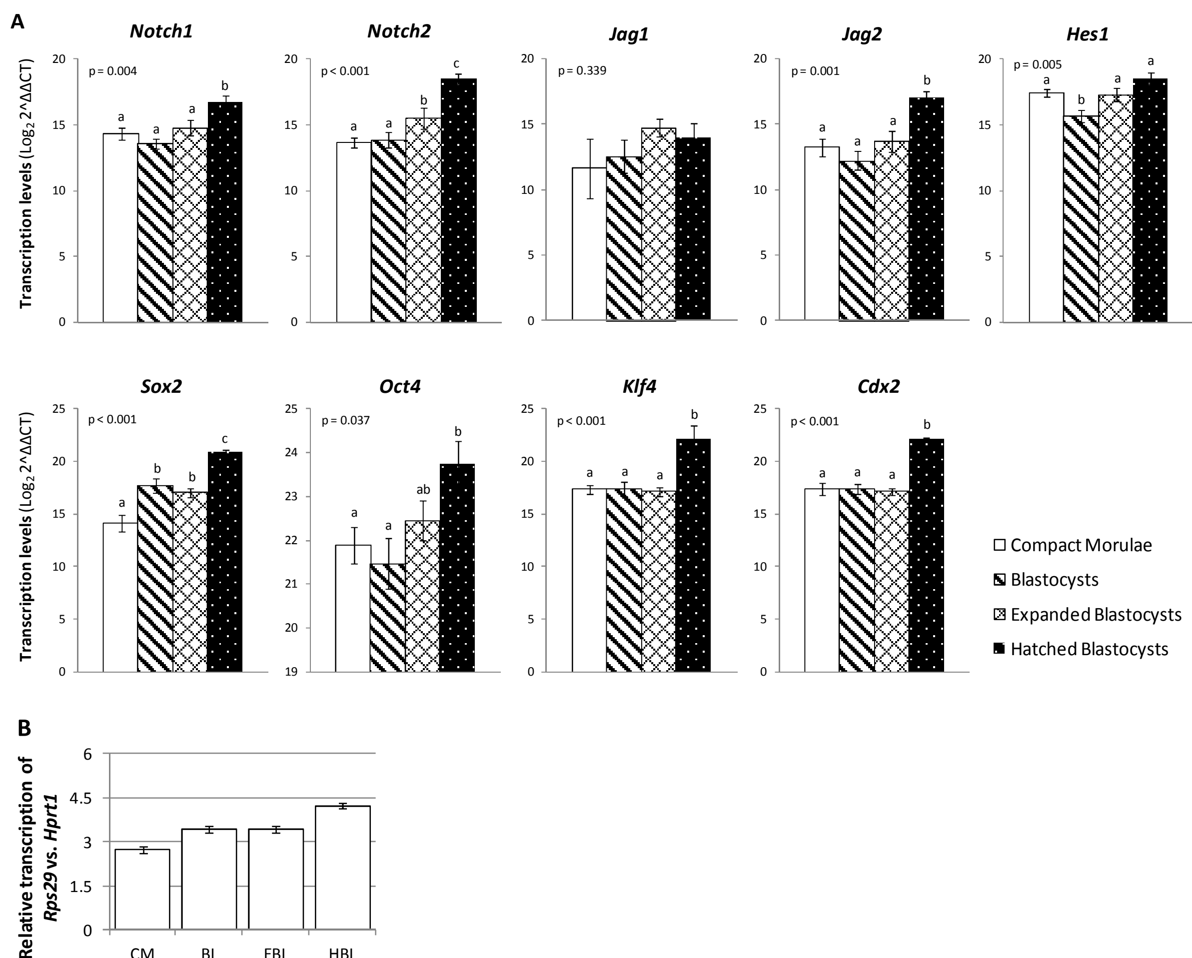


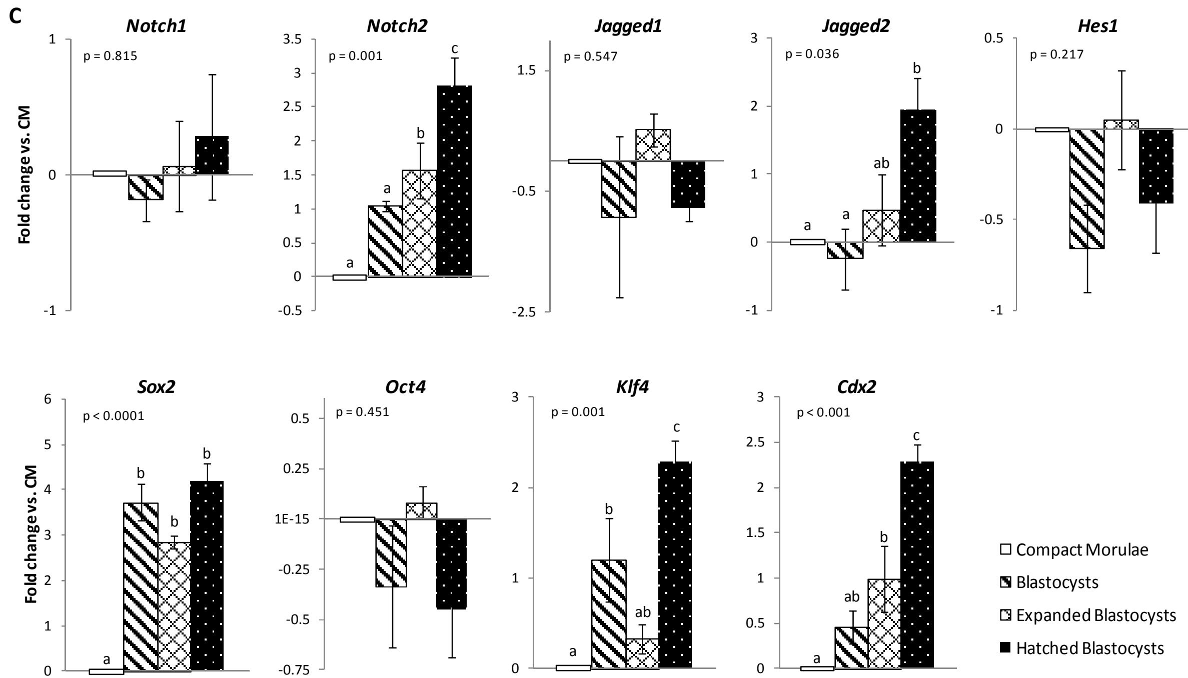
Agarose gels of qRT-PCR products. For each Notch component gene (receptors, ligands and effectors; plus negative control), four representative embryos of each developmental stage (3.5 dpc compact morulae – CM, blastocysts – BL and expanded blastocysts – EBL, and 4.5 dpc hatched blastocysts – HBL) are shown. For each pluripotency and differentiation gene markers, two representative embryos of each developmental stage are shown. Ladder: DNA ladder with 50 bp increments; the arrow (→) signals the 50 bp mark; C+: positive control gene; for each analyzed gene, a tissue sample known to transcribe the analyzed gene was added, and the qRT-PCR reaction product added in the gel (see Methods section for details); NTC: non-template control.

Figure 5A, shows the mean transcription levels of Notch and pluripotency and differentiation genes at each developmental stage (values are presented as the Log_2 of

power of ΔΔCt values). Only genes with consistent transcription among embryos were considered in this analysis. Figure 5B shows the fold change values of transcription levels of *Rps29* and *Hprt1* control endogenous (housekeeping) genes at each developmental stage. The transcription levels of target genes at the BL, EBL and HBL stages were then compared to those at the CM stage (values are presented as the Log_2 of power of ΔΔCt values, with CM stage as calibrator) (Figure 5C). Based on above results, the dynamics of gene transcription along developmental stages is schematically illustrated in Figure 6. As depicted from these figures, transcription of *Sox2*, *Oct4*, *Klf4*, *Cdx2*, *Notch1*, *Notch2* and *Jagged2* increased throughout development, mainly at the HBL stage, whereas transcription of *Jagged1* and *Hes1* remained fairly constant.

Figure 5: Transcription of Notch components and pluripotency and differentiation gene markers in mouse early embryonic development.





Quantitative real-time (qRT-PCR) was used to detect and quantify the presence of transcripts in 3.5 dpc compact morulae (n=9), blastocysts (n=9) and expanded blastocysts (n=7), and in 4.5 dpc hatched blastocysts (n=5). Analyzed genes (most prevalent): Notch receptors – *Notch1* and *Notch2*; Notch ligands – *Jagged1* and *Jagged2*; Notch effectors – *Hes1*; Pluripotency and differentiation marker genes – *Sox2*, *Oct4*, *Klf4* and *Cdx2*. Bars represent mean transcription levels ± s.e.m. ANOVA *p* values are indicated for each gene analysis. Bars with different letters differ significantly (*post-hoc* LSD). A: For data analysis, Ct values were normalized to housekeeping gene 1 (*Rps29*) and the ΔCt values obtained further calibrated with housekeeping gene 2 (*Hprt1*), generating ΔΔCt values. These values were log transformed and results presented as the Log2 of power of ΔΔCt values. B: Log2 of power of ΔCt values of transcription levels of housekeeping genes *Rps29* and *Hprt1* at each developmental stage; CM = Compact Morulae; BL = Blastocyst; EBL = Expanded Blastocyst; HBL = Hatched Blastocyst. C: For data analysis, Ct values of each target gene were normalized with the mean Ct values of housekeeping genes *Rps29* and *Hprt1*, and the obtained ΔCt values were then calibrated to ΔCt values of compact morulae (shown as 0.0), originating the ΔΔCt values for log transformation. Results are also presented as the Log2 of power of ΔΔCt values.

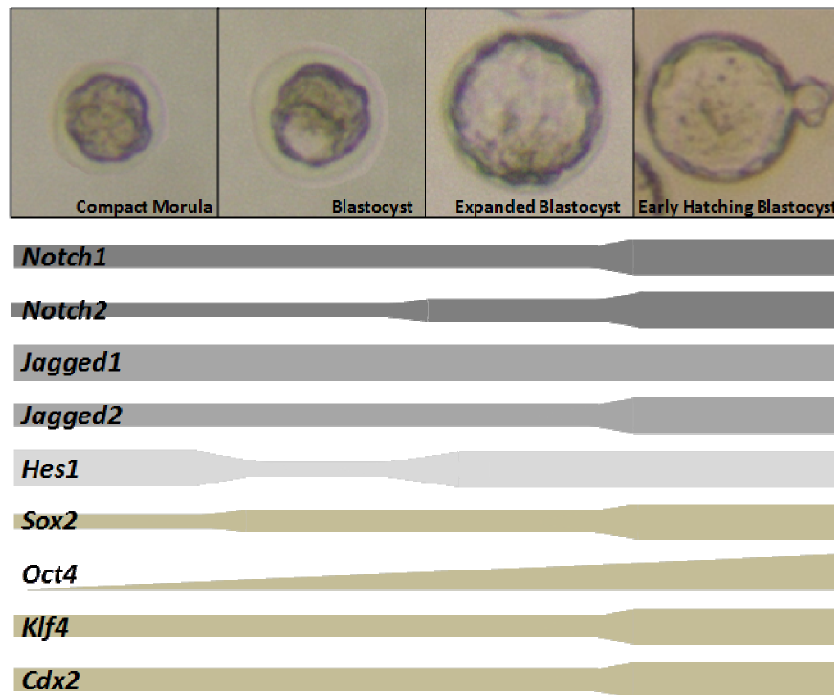


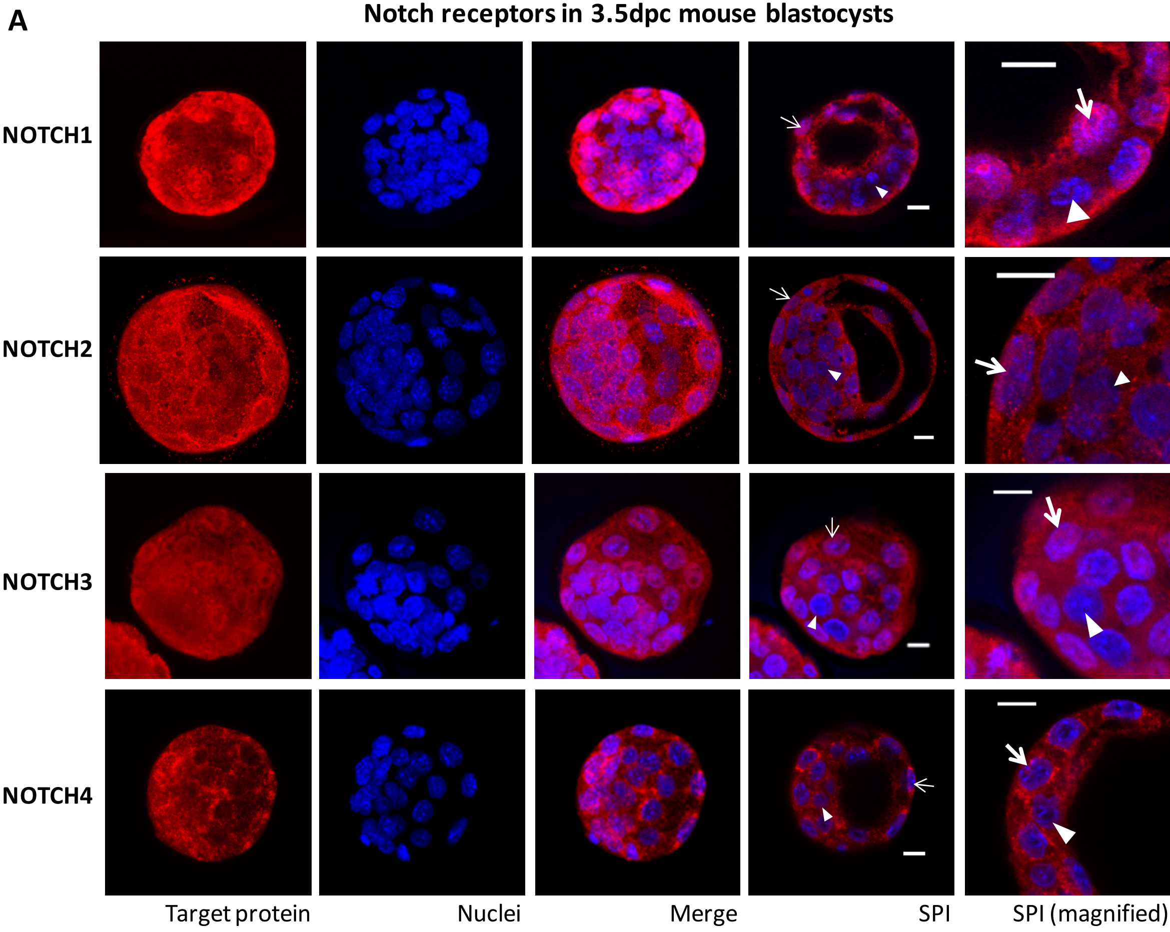
Figure 6: Schematic illustration of the dynamic transcription patterns of Notch and pluripotency and differentiation genes along mouse early embryonic development.

Transcription levels of *Notch1*, *Notch2*, *Jagged2* and *Hes1* correlated with those of all pluripotency and differentiation genes (r= 0.72 to 0.95, *p*= 0.004 to *p*< 0.0001). *Notch1* correlated with *Notch2*, *Jagged2* and *Hes1* (r= 0.75 to 0.86, *p*< 0.0001), *Notch2* correlated with *Jagged2* and *Hes1* (r= 0.79 and 0.72, *p*< 0.0001 and *p*= 0.001, respectively), and *Jagged2* correlated with *Hes1* (r= 0.78, *p*< 0.0001).

3.1.4.2. Gene expression

Since the BL represents the earliest developmental stage in which the two initial cell lineages – ICM and TE – are segregated and have reached their final spatial location, this embryonic stage was chosen to evaluate the presence of Notch proteins. As shown in Figure 7A, NOTCH1-4 were expressed in BL, and NOTCH1 and NOTCH3 were detected in the nucleus of presumptive TE cells. This indicates that the receptors were cleaved and NICD was translocated into the nucleus, thus confirming Notch signaling activation through these receptors. Ligands DLL4 and JAGGED1 were expressed in BL, whereas ligands DLL1 and JAGGED2 were not detected (Figure 7C). Effector HES1 was detected in the nucleus of some cells, whereas HES2 only showed a diffuse pattern in the cytoplasm (Figure 7B).

Figure 7: Expression of Notch components in mouse blastocysts



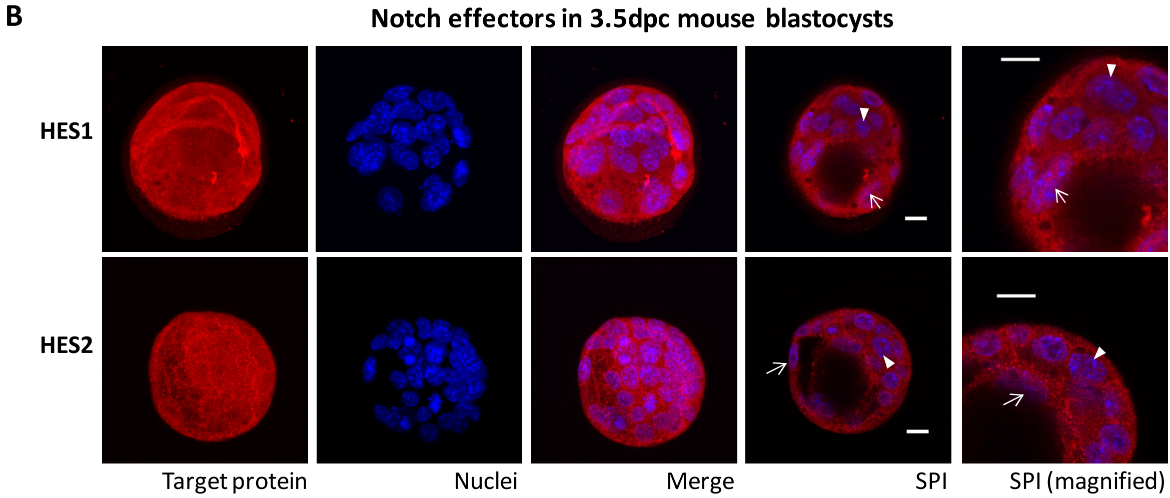
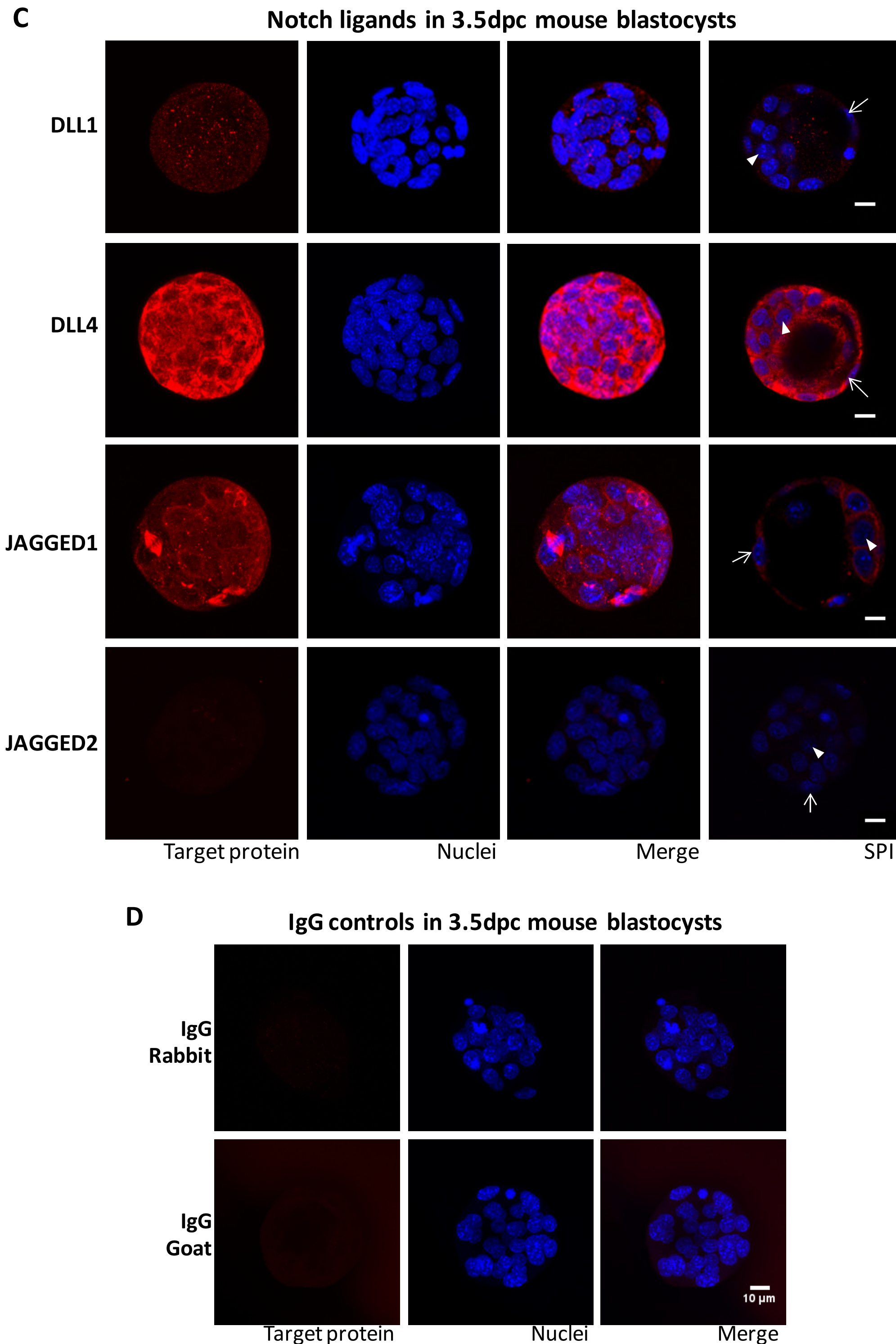


Figure 7 (continuation)



Expression of Notch receptors NOTCH1-4 (A), Notch effectors HES1-2 (B), Notch ligands Delta-like1 and 4 (DLL1 and 4) and JAGGED1-2 (C), and negative controls (Rabbit and Goat IgG; D) in 3.5 dpc blastocysts. Confocal photomicrographs show representative images of each target protein immunostaining. Images were selected to show the similar staining pattern of six blastocysts, for each protein. Target proteins are stained red and nuclei are stained blue with Hoechst. Images in the first three columns are maximum intensity projections of the obtained Z-stack; the fourth and fifth columns are representative single plane images (SPI). Examples of presumptive trophectoderm cells' nuclei are marked with arrows (→) and examples of presumptive inner cell mass cells' nuclei are marked with arrowheads (►). Scale bar 10µm. Notice that there is no detectable staining for DLL1 and JAGGED2 proteins, which show a similar staining to that of negative controls.

3.1.4.3. Notch signaling activation or blockade in cultured embryos

To further confirm Notch activity in mouse early embryonic development, Notch signaling was inhibited through a pharmacological approach with DAPT, a γ-secretase inhibitor which prevents the intracellular cleavage of NICD and its translocation to the nucleus. Together with embryo culture supplementation with recombinant JAGGED1 and JAGGED2 (putative Notch activators), this experiment allowed the observation of effects of Notch signaling inhibition or activation on blastocyst development and hatching. As shown in Table 6, DAPT, JAGGED1 and JAGGED2 treatments had no effect on embryo viability, as depicted from the number of non-degenerated morphologically normal embryos progressing in culture. However, embryo kinetics was affected by DAPT treatment, which decreased the early hatching blastocyst rate at 4.0 dpc ($p< 0.05$), and the hatched blastocyst rate at 4.5 dpc (statistical tendency, $p= 0.12$) (Figure 8B-C). At 4.0 dpc, both JAGGED1 and JAGGED2 treatments prevented the progression of CM (Figure 8B), whereas at 4.5 dpc JAGGED2 supplementation significantly inhibited blastocyst hatching (Figure 8C).

Table 6: Effect of embryo culture supplementation with DAPT, JAGGED1 and JAGGED2 on mouse embryo survival.

Group	n	3.5 dpc embryos n (%)	n	4.0 dpc embryos n (%)	n	4.5 dpc embryos n (%)
Control	216	200 (93%)	192	176 (92%)	127	114 (90%)
DAPT	86	77 (90%)	88	78 (89%)	26	21 (81%)
JAGGED1	146	131 (90%)	128	120 (94%)	108	95 (88%)
JAGGED2	102	97 (95%)	103	99 (96%)	40	32 (80%)

Columns marked as n show the total number of embryos present in culture; columns marked as 3.5 dpc, 4.0 dpc and 4.5 dpc show the number of non-degenerated morphologically normal embryos progressing in culture up to that time-point.

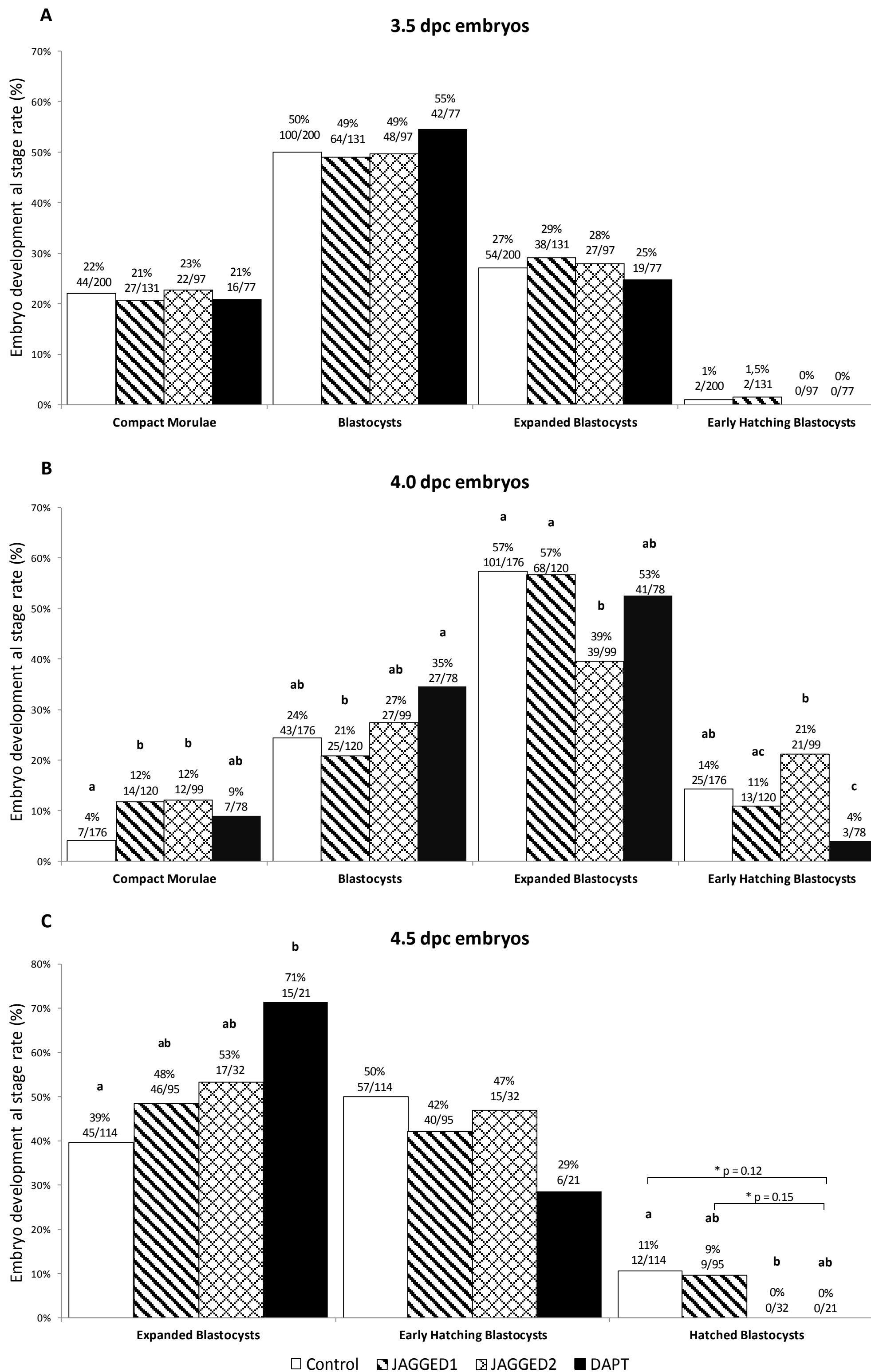


Figure 8: Effect of pharmacological Notch signaling inhibition and activation on mouse embryo developmental kinetics.

Mouse 2.5 dpc embryos were *in vitro* cultured in the presence of a Notch inhibitor (DAPT) or Notch ligands JAGGED1 and JAGGED2 until 4.5 dpc. Embryos were observed after 24 hours in culture (at 3.5 dpc; A), 36 hours in culture (at 4.0 dpc; B) and 48 hours in culture (at 4.5 dpc; C) and morphologically evaluated. *In vitro* culture of a subset of embryos was discontinued at 3.5 dpc or 4.0 dpc to perform transcription analysis. Numbers above bars indicate the number of viable embryos in culture / the number of total embryos. Different letters within the same developmental stage differ significantly, $p< 0.05$ (Chi-square test). Asterisks (*) indicate the exact p value of the Chi-square test.

To evaluate the possible relationship between the above changes in developmental kinetics and gene transcription, individual EBL of control and treated groups were analyzed by qRT-PCR for transcription of *Notch1, Notch2*, *Jagged1*, *Jagged2*, *Hes1*, *Sox2*, *Oct4*, *Klf4*, *Cdx2*, *Lgr5* and *Cdca7*. Transcription of this latter pluripotency gene (*Cdca7*), regulated by Notch in later embryonic events, such as hematopoietic stem cell emergence (Guiu et al. 2014) was here detected at this earlier stage of development. As shown in Figure 9 (A-J), Notch signaling blockade by DAPT downregulated transcription of *Hes1* and *Sox2* (p< 0.0001) and tended to decrease (p= 0.06) transcription of *Notch2*. Supplementation with JAGGED1 decreased *Jagged1* transcription (although non-significantly) and had no effect on *Jagged2* transcription. In contrast and interestingly, supplementation with JAGGED2 although not affecting its own transcription, abolished *Jagged1* transcription in all but one embryo, and downregulated *Cdx2* transcription. The presence of transcripts of *Jagged1*, *Jagged2* and *Cdx2* following treatments with DAPT, JAGGED1 and JAGGED2 was further confirmed by qRT-PCR product visualization in agarose gels (Figure 9 K). Additionally, the transcription of the negative control *Lgr5* was not detected.

Figure 9: Effect of pharmacological Notch signaling inhibition and activation on gene transcription in 4.0 dpc mouse expanded blastocysts

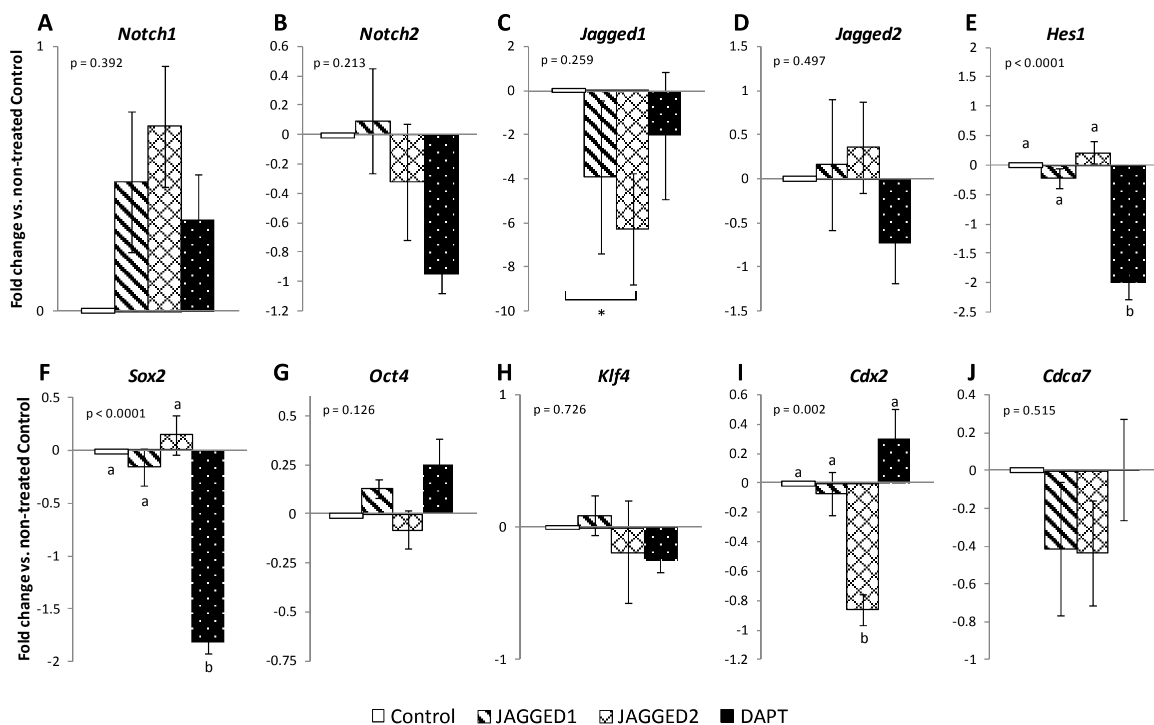
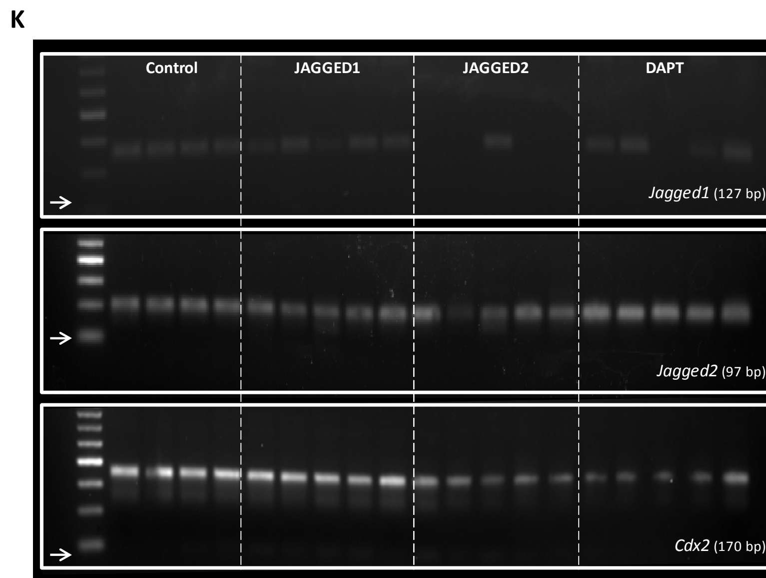


Figure 9 (continuation)



Mouse 2.5 dpc embryos were *in vitro* cultured in the presence of a Notch inhibitor (DAPT) or of Notch ligands JAGGED1 and JAGGED2, for 36 hours, until 4.0 dpc. Expanded blastocysts from groups Control (n=5), JAGGED1-treated (n=5), JAGGED2-treated (n=5) and DAPT-treated (n=6) were processed for qRT-PCR analysis. A-J: Transcription of *Notch1* (A), *Notch2* (B), *Jagged1* (C), *Jagged2* (D) and *Hes1* (E), and of pluripotency and differentiation genes *Sox2* (F), *Oct4* (G), *Klf4* (H), *Cdx2* (I) and *Cdca7* (J) were analyzed. Bars represent Log_2 of power of ΔΔCt values. These values were generated by first normalizing the Ct values of each target gene with the mean Ct values of the endogenous control genes *Rps29* and *Hprt1*, at each embryonic developmental stage. The obtained ΔCt values were then calibrated to ΔCt values of Control embryos, which were used as calibrators (shown as 0.0), originating the ΔΔCt values for log transformation; error bars show the standard error of the mean (s.e.m). Exact ANOVA results (*p*) are shown for each gene. Different letters within the same gene represent significantly different mean values ($p < 0.05$; LSD post-hoc). *Transcription of *Jagged1* differs significantly (*p*= 0.038) between groups Control and JAGGED2-treated (T-test). K: Agarose gels of qRT-PCR products of genes *Jagged1*, *Jagged2* and *Cdx2*. Images illustrate results from representative 4.0 dpc expanded blastocysts from groups Control (n= 4), JAGGED1-treated (n= 5), JAGGED2-treated (n= 5) and DAPT-treated (n= 5). The DNA ladder has 50 bp increments, and the arrow (→) signals the 50bp mark.

Correlation analysis showed that control 4.0 dpc EBL showed a positive strong correlation between *Hes1* and *Cdca7* (r = 0.98; *p*= 0.005). A similar correlation was found in DAPT treated embryos (r = 0.98; *p*= 0.001), but was not present in JAGGED1 (*p*= 0.32) and JAGGED2 (*p*= 0.32) supplemented embryos.

3.1.5. Discussion

To the author's best knowledge, this is the first report on the dynamics of transcription of Notch and of markers of embryonic pluripotency and differentiation genes in individual embryos, from the time of the first cellular differentiation to blastocyst hatching. The results indicate that transcription of Notch components is highly dynamic during mouse blastocyst development and hatching. This approach allowed the assessment of gene transcription

relationships, at the individual embryo level, providing so far unique data, not available from studies with pools of embryos or isolated blastomeres. This individual embryo approach revealed that transcription of *Notch1*, *Notch2*, *Jagged1*, *Jagged2* and *Hes1* was ubiquitous from the CM to HBL stages, whereas transcription of *Notch3*, *Notch4*, *Dll4* and *Hes2* was inconsistent along those developmental stages. These transcription patterns of Notch genes partially deviate from those reported by Cormier et al. (2004), who evaluated transcription in pools of mouse embryos by nested RT-PCR (inconsistencies between studies in *Notch3*, *Notch4*, *Dll1* and *Dll4* transcription patterns). In the present study, the high accuracy and sensitivity of qRT-PCR, as well as the confirmation of amplicon sequence, allowed for the exclusion of false positives resulting from unspecific amplifications of similar strands of nucleotides, as well as the detection of very small amounts of mRNA copies from single embryos. Recent studies used RNA-Seq analysis of single mouse blastomeres to identify several species of mRNA (Tang et al. 2011; Deng et al. 2014). Although this is a very useful approach to evaluate overall transcription status of a given cell, a full scan of the whole embryonic cells, especially of more advanced stages, such as HBL which can comprise up to 70 cells (Montag et al. 2000), is still not available. Since intercellular communication requires the analysis of both the signal sending and signal receiving cells, the loss of information from either of these cells, will provide an incomplete picture of embryonic gene transcription. In fact, in the above studies (Tang et al. 2011; Deng et al. 2014) a low number of copies of *Notch2*, *Dll4, Jagged1, Jagged2* and *Hes2* transcripts were detected, or were not detected at all. This could be due not only to individual embryo variability, as also observed by others (Menchero et al. 2019), but also to the individual blastomere signaling status, which could be in either a signal sending or signal receiving state, since they are mutually exclusive (Sprinzak et al. 2010).

The presence of transcripts in embryos needs to be interpreted with caution, since an oocyte mRNA pool may be present and be responsible for protein production before the activation of the embryonic genome (Bianchi and Sette 2011). Although most of this mRNA pool is translated into protein and degraded during maternal to embryonic transition, which in the mouse occurs mainly at the 2-cell stage (Latham et al. 1991), up to 10% of maternal mRNA persists until the BL stage (Bell et al. 2008). Additionally, cells have post-transcriptional regulating mechanisms that allow them to stock mRNA without immediately translating it into protein (Harvey et al. 2017). This means that the presence of transcripts may not reflect the protein composition of an embryo at a given stage. In fact, in BL, although *Notch4* and *Dll4* transcripts were not detected, NOTCH4 and DLL4 proteins were detected. These proteins may have been translated at previous embryonic stages and have not yet been degraded. Inversely, *Jagged2* transcripts were detected in all embryos, but JAGGED2

protein was not expressed in BL. At this stage, the embryo may be merely storing *Jagged2* mRNA, which will be translated at a later stage. In fact, the translation of the accumulated *Jagged2* transcripts may only occur at hatching when the embryo enters in direct contact with the endometrium. In this scenario, JAGGED2 may be involved, both in the hatching process and in embryo-maternal communication, since several Notch receptors and effectors were identified in the mouse uterine epithelium (Murta et al. 2015).

Notch1 transcription was constant until the EBL stage, increasing at the HBL stage, and signaling was activated through this receptor, as the protein was detected in the nucleus. This may indicate that besides a constitutive function (Chu et al. 2011; Rayon et al. 2014; Menchero et al. 2019) NOTCH1 may be regulating other cell functions. NOTCH3 was also detected in the nucleus of embryonic cells, which indicates that Notch signaling is also being activated through this receptor. On the other hand, as NOTCH2 remains in the cytoplasm and, since Notch receptors are not redundant (Cheng et al. 2007), results indicate that, at this embryonic stage, only NOTCH1 and NOTCH3 are being required. Effector HES1 was detected in the nucleus, whereas HES2 only showed a diffuse staining pattern in the cytoplasm. This indicates that Notch signaling may be conveyed through HES1. In this scenario, as JAGGED2 is not expressed at the BL stage, JAGGED1 appears as the ligand involved in canonical Notch receptor activation. The diffuse pattern accumulation of HES2 in the cytoplasm after translation, without translocation to the nucleus, was already observed in other scenarios (Zheng et al. 2008). This may indicate an additional regulatory mechanism for conveying Notch activity in embryos. Further studies are required to investigate the participation of other Notch effectors, such as the Hey gene family (Fischer and Gessler 2007), or if Notch signaling is established non-canonically, namely by interacting with other signaling pathways such as Wnt (Andersen et al. 2012) and Hippo (Rayon et al. 2014; Watanabe et al. 2017; Menchero et al. 2019).

Transcription of embryonic pluripotency and differentiation gene markers followed the patterns previously described by others (Wang et al. 2004; Guo et al. 2010; Tang et al. 2011). The transcription levels of these genes were correlated with those of Notch genes, suggesting that they may be the target of Notch signaling or, conversely, operate to modulate Notch signaling. Menchero et al. (2019) showed that Notch is a major activator of *Cdx2* transcription from the 2-cell to the morula stage, but from this stage until the blastocyst stage, *Cdx2* transcription is activated by Hippo. In this study, transcription of *Cdx2* correlated with those of *Notch1*, *Notch2*, *Jagged2* and *Hes1*. Therefore, Notch may still be regulating *Cdx2* transcription at the BL stage.

The presence of Notch signaling activity was further confirmed by the observed downregulation of its effector *Hes1*, following DAPT treatment. This pharmacological blockade of Notch signaling affected embryo developmental kinetics, retarding blastocyst hatching, and downregulated *Sox2* transcription. The above effect on blastocyst hatching was also observed following JAGGED2 supplementation. This indicates that a deregulation in Notch signaling, either by its over or under-activation, affects blastocyst development and hatching.

Modulation of Notch signaling through its serrated type ligands has been widely used in many pathological scenarios. The use of anti-JAGGED or JAGGED overexpression therapies has been extensively studied with varying results (Kared et al. 2006; Li et al. 2014; Vieira et al. 2015). JAGGED1 supplementation had no major effect on embryo developmental kinetics. This could be due to a sufficient expression of this ligand by the embryo itself, turning supplementation redundant. Interestingly, JAGGED2 supplementation abolished *Jagged1* transcription, and this was associated with a downregulation of *Cdx2* transcription and with an impaired blastocyst hatching. This indicates that JAGGED2 supplemented embryos had no internal or external source of JAGGED1 to maintain a satisfactory *Cdx2* transcription level. This points to a regulatory mechanism by which JAGGED1 controls *Cdx2* transcription, and the completion of blastocyst hatching. Alternatively, as CDX2 is not believed to be an active participant in this process (Rayon et al. 2014), it is possible that *Jagged1* is linked to blastocyst hatching through its interplay with other cell signaling pathways.

Notch signaling activates *Cdca7* transcription in hematopoietic stem cell specification during zebrafish embryonic development (Guiu et al. 2014). Here, in the mouse model, transcription of *Cdca7* was ubiquitously detected at a much earlier embryonic developmental stage. Transcription of *Cdca7* correlated with that of *Hes1*, in control and DAPT-treated embryos. Both *Hes1* and *Cdca7* have promoters with RBPJ-binding sites, being potential Notch transcriptional targets. However, transcription of *Hes1* was downregulated by DAPT treatment, whereas *Cdca7* transcription was not affected. This may indicate that *Notch* is not regulating *Cdca7* transcription in this mammalian embryonic stage scenario. Nevertheless, the observed significant correlation between *Hes1* and *Cdca7* deserves further investigation.

3.1.6. Conclusions

In conclusion, this study characterized the transcription and expression of Notch pathway components (receptors, ligands and effectors) at the individual embryo level, during

mouse blastocyst development and hatching. The transcription levels of Notch genes followed a dynamic pattern along development. Transcription levels of *Notch1*, *Notch2*, *Jagged2* and *Hes1* correlated with each other and with those of pluripotency and differentiation genes. Gene transcription was associated to protein expression, except for JAGGED2, where high transcription levels in all embryos were not translated into protein, possibly reflecting mRNA storage for use at a later stage of development and/or interaction with the endometrium. Presence of Notch signaling activity was confirmed through nuclear NICD and HES1 detection, and downregulation of *Hes1* transcription following canonical signaling blockade with DAPT. Data lead to the suggestion that Notch canonical signaling may be operating through NOTCH1, NOTCH3, JAGGED1 and HES1. *In vitro* embryo culture supplementation with JAGGED1 had no effect on embryo developmental kinetics. In contrast, supplementation with JAGGED2 abolished *Jagged1* transcription, downregulated *Cdx2* transcription and inhibited blastocyst hatching. This unveiled a possible regulatory effect between JAGGED1, CDX2 and blastocyst hatching. Notch signaling blockade by DAPT downregulated transcription of *Sox2*, and retarded embryo hatching. This indicates that a deregulation in Notch signaling, either by its over or under-activation, affects blastocyst development and hatching.

3.2. Chapter II – Early embryo balanced Notch and Wnt signaling interplay is required for embryo and fetal development to term

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Submitted to: *Reproduction**

3.2.1. Abstract

This study investigated the role of Notch and Wnt cell signaling interplay in the mouse early embryo, and its effects on fetal development. Developmental kinetics was evaluated in embryos *in vitro* cultured from the 8-16-cell to the hatched blastocyst stage in the presence of signaling inhibitors of Notch (DAPT) and/or Wnt (DKK1). An embryo subset was evaluated for differential cell count and gene transcription of Notch (receptors *Notch1-4*, ligands *Dll1*, *Dll4*, *Jagged1-2*, effectors *Hes1-2*), Wnt (ligand *Wnt3a*, co-receptor *Lrp6*) and pluripotency and differentiation markers (*Sox2*, *Oct4*, *Klf4*, *Cdx2*), whereas a second subset was evaluated for implantation ability and development to term following transfer into recipients.

Notch and Wnt blockades had significant opposing effects on developmental kinetics – Notch blockade retarded while Wnt blockade fastened development. This evidences that Notch and Wnt regulate the pace of embryo kinetics by respectively speeding and braking development. Blockades significantly changed the transcription profile of *Sox2*, *Oct4*, *Klf4* and *Cdx2*, and Notch and double blockades significantly changed embryonic cell numbers and cell ratio. Double blockade induced more severe phenotypes than expected by cumulative effects of single blockades. Implantation ability was unaffected by treatment, but Notch and double blockades significantly decreased fetal development to term. Compared to control embryos, Notch blockade and Wnt blockade embryos originated, respectively, significantly lighter and heavier fetuses.

In conclusion, Notch and Wnt signaling interplay in the regulation of the pace of early embryo kinetics, and their actions at this stage have significant carry-over effects on later fetal development to term.

* Text adapted from the original submitted paper

3.2.2. Introduction

Mammalian preimplantation embryo development considers highly regulated events of cell proliferation and differentiation. The first cellular differentiation, which in the mouse embryo begins at the 16-cell stage, originates two cell lineages: i) the trophectoderm (TE), the outer layer of the blastocyst, which will physically interact with the endometrium and constitute the fetal part of the placenta; and ii) the inner cell mass (ICM), located inside the TE, which will differentiate into the epiblast (embryo itself) and the primitive endoderm (Kwon et al. 2008; Marikawa and Alarcon 2012). Each cell lineage expresses distinct marker proteins. TE cells express CDX2, which downregulates expression of the pluripotency factors OCT4 and Nanog (Strumpf et al. 2005), whereas pluripotent ICM cells express OCT4 and Nanog, which together with SOX2 are responsible for maintaining their undifferentiated state (Schrode et al. 2013). This cellular lineage commitment and specification relies on cell polarity establishment and intercellular communication (Rossant and Tam, 2009) established through a complex signaling network (Zhang et al. 2007, Hayward et al. 2008). This network includes Notch and Wnt cell signaling pathways (Cormier et al. 2004, Kemp et al. 2005, Xie et al. 2010, Aghajanova et al. 2012) as regulators of pluripotency and differentiation transcription factors (Marson et al. 2008; van den Berg et al. 2010; Kelly et al. 2011; Neves et al. 2011; Huang et al. 2015, Batchuluun et al. 2017).

In the mouse, Notch is composed of four receptors (Notch1-4) and five ligands (Delta-like – DLL1, 3 and 4 and Jagged1-2). Canonical Notch signaling is initiated by the binding of a ligand expressed on the surface of one cell (signal-sending) to the extracellular domain of the receptor expressed on a neighboring cell (signal-receiving). This leads to the cleavage of the receptor by a series of enzymes, including a γ-secretase complex, and the release of the Notch intracellular domain (NICD), which is then translocated to the nucleus where it associates with the DNA binding transcription factor RBPJ to regulate transcription of Notch effector genes (including *Hes1*, *Hes2* and *Hes5*) (Borggrefe and Oswald 2009; Andersen et al. 2012). Non-canonical Notch signaling can be ligand dependent or independent and does not require NICD interaction with RBPJ (Andersen et al. 2012). Earlier studies in the mouse reported that canonical Notch signaling (RBPJ-dependent) is dispensable for early embryo development (Shi et al. 2005; Souilhol et al. 2006), but subsequent studies showed that canonical signaling inhibition with DAPT (a γ-secretase inhibitor) decreased embryo hatching (Batista et al. 2020) and implantation (Chu et al. 2011). Also, studies using mutant knockout embryos evidenced a role for Notch signaling on TE lineage assignment (Rayon et al. 2014; Watanabe et al. 2017; Menchero et al. 2019).

Wnt signaling may operate through canonical (β-catenin dependent) and non-canonical variants (Kim et al. 2013). Canonical Wnt signaling is initiated by the interaction of Wnt ligands with Frizzled receptors (Fzd) and LRP5/6 co-receptors. This leads to the stabilization of cytoplasmic β-catenin and its translocation to the nucleus where it binds with transcription activators, promoting the transcription of Wnt target genes (Rao and Kuhl 2010). In the mouse, *in vivo* blockade of canonical Wnt signaling, although not affecting development until the blastocyst stage, decreased implantation rate following embryo transfer to recipients (Xie et al. 2008). Interestingly, the role of Wnt signaling on embryonic development appears to be species-dependent. In the bovine, *in vitro* blockade of canonical Wnt signaling, as in the mouse, had no effect on blastocyst development (Denicol et al. 2013), but instead improved later pregnancy establishment (Denicol et al. 2014), and the up-regulation of canonical Wnt signaling decreased the *in vitro* bovine blastocyst rate and cell number (Denicol et al. 2013). By contrast, in pigs, *in vitro* Wnt canonical signaling blockade increased blastocyst hatching ability as well as TE and total cell number (Lim et al. 2013).

The above studies evidenced that Notch and Wnt signaling early in development affected later blastocyst competence to implant. Disruption of early embryonic development cellular events may originate carry-over effects upon fetal development, ultimately leading to a non-viable fetus and/or neonate (Noli et al. 2015; Almagor et al. 2016). Notch and Wnt signaling are simultaneously active in several embryonic (Hayward et al. 2008) and adult tissues (Muñoz Descalzo and Martinez Arias 2012) in a cross-regulatory manner, namely in cell fate determination of progenitor and stem cells and in cancer development, either in a cooperative or counteractive manner (Andersen et al. 2012; Collu et al. 2014). The existence of such Notch-Wnt interplay in preimplantation embryo development is unknown. This study evaluated Notch-Wnt interplay in mouse early embryo development, and its role on subsequent implantation and pregnancy maintenance to term.

3.2.3. Materials and Methods

3.2.3.1. Animals

Experimental procedures were conducted according to the national and European Union legislation (Directive 2010/63/UE) regarding the use of animals for experimental purposes, and under license of the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal; Ref. 001/2018). Mice (*Mus musculus*; Crl: CD1

(ICR) (CD1)) were purchased from Charles River Laboratoire France, and housed in standard facilities at Faculdade de Medicina Veterinária, Universidade de Lisboa (Lisbon, Portugal). Mice were maintained in a 12 h light/dark cycle, in corn cob bedded cages and with ad libitum access to standard laboratory diet and water. Mice health was monitored daily.

3.2.3.2. Embryo recovery and *in vitro* culture

Embryos were recovered from 2-3 months-old females, according to procedures previously described (Batista et al. 2020). Briefly, females were superovulated with equine chorionic gonadotropin (10 IU; Intergonan; MSD Animal Health, Portugal) and human chorionic gonadotropin (10 IU; hCG; Chorulon; MSD Animal Health), and housed overnight with a male. At 2.5 dpc, females were euthanized by cervical dislocation under general anesthesia (150mg kg^{-1} ketamine + 10mg kg^{-1} xylazine) and embryos collected by oviduct flushing with M2 medium (Sigma-Aldrich, St Louis, MO, USA). Embryos at the 8-16-cell stage were selected, washed in fresh M2 medium, randomly allocated to treatment groups and *in vitro* cultured in 500µL of KSOM (Millipore, Specialty Media, Germany) overlaid with 400µL of mineral oil (EmbryoMax®, Millipore) in 4-well dishes (Nunclon, Nunc, Roskilde, Denmark), at 37^{0}C in a 90%N_2+5%O_2+5%CO_2 humidified atmosphere.

3.2.3.3. Experimental design

This study tested two hypothesis: i) Notch and Wnt signaling interplay regulates the pace of early embryo development; and ii) Notch and Wnt interplay in the early embryo has carry-over effects on later embryo-fetal development. To address these hypotheses, Notch, Wnt and Notch plus Wnt canonical signaling blockades were started before, and kept during the first embryonic differentiation, i.e. from the early morula to the expanded blastocyst stages. This allowed to test the following: i) if Notch and Wnt regulate the first embryonic differentiation, then the signaling blockade will affect the physiologic pace of development; ii) if Notch and Wnt interplay, then each signaling blockade will affect signaling of the other and the physiologic pace of development; and iii) if Notch-Wnt regulated cellular events at the first embryonic differentiation are critical for later stages, then signaling blockade of each or both pathways will disrupt subsequent physiologic development.

Notch canonical signaling blockade was achieved by supplementing KSOM with 100 µM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; Sigma-

Aldrich), a γ-secretase inhibitor which prevents the cleavage of NICD and consequently its translocation to the nucleus, as used in many *in vitro* (Jiang et al. 2011) and *in vivo* (Murta et al. 2014; Batista et al. 2020) scenarios. Wnt canonical signaling blockade was achieved by supplementing KSOM with 100 ng mL^{-1} DKK1 (Dickkopf-1; 5897-DK-010/CF, R&D Systems, Bio-Techne, Minneapolis, USA) (Denicol et al. 2013), which binds to Wnt co-receptors (LRP5/6), preventing ligand-receptor interaction and promoting LRP5/6 endocytosis, thus inhibiting β-catenin stabilization and nuclear translocation (Li et al. 2010). Double blockade was achieved by supplementing KSOM with both 100 µM DAPT and 100 ng mL^{-1} DKK1.

In each of 27 *in vitro* culture sessions, selected 8-16-cell embryos were randomly allocated, in groups of 20, to: i) group Control: KSOM supplemented with DAPT vehicle (dimethyl sulfoxide, DMSO; Sigma-Aldrich) and DKK1 vehicle (phosphate-buffered saline – PBS with 0.1% (w/v) bovine serum albumin, BSA; Sigma-Aldrich); ii) group DAPT (Notch blockade group): KSOM supplemented with DAPT; iii) group DKK1 (Wnt blockade group): KSOM supplemented DKK1; and iv) group DAPT+DKK1 (double blockade): KSOM supplemented with DAPT and DKK1. In 6 of the 27 *in vitro* culture sessions, embryos were cultured until 4.5 dpc and morphologically evaluated for quality and developmental stage (compact morula – CM; blastocyst – BL; expanded blastocyst – EBL; hatched blastocyst – HBL) (Nagy *et al.*, 2003) at 3.5 dpc (after 24 hours in culture), 4.0 dpc (after 36 hours in culture) and 4.5 dpc (after 48 hours in culture). In 10 of the 27 *in vitro* culture sessions, embryos were cultured until 3.5 dpc, at which time they were morphologically evaluated and processed either for differential cell count or gene transcription analysis (components of Notch and Wnt pathways and markers of embryonic pluripotency and differentiation). In the remaining 11 *in vitro* culture sessions, embryos were morphologically evaluated at 3.5 dpc and 4.0 dpc, at which time they were transferred to pseudo pregnant recipients and implantation rate, fetal sex and weight at term were evaluated.

3.2.3.4. Embryo differential cell count

Embryos were fixated in 4% (w/v) paraformaldehyde in PBS for 30 min at 4^{0}C, washed in PBS, permeabilized in 0.5% (v/v) TritonX-100 in PBS for 1 min, and again washed in PBS. Blocking was performed with 2.5% (w/v) BSA (Sigma-Aldrich) in 0.1% (v/v) Tween®20 in PBS for 1 h at room temperature. Embryos were then incubated over-night at 4^{0}C with primary antibody rabbit anti-CDX2 (ab88129, Abcam, Cambridge, UK) diluted in blocking solution (1:200). After washing in PBS (4 × 5 min), embryos were incubated with AlexaFluor® 594 secondary antibody (A11012, Life Technologies, Thermo Fischer Scientific,

USA) diluted in blocking solution (1:300), for 30 min at room temperature. After a 2 × 10 min wash in PBS, nuclei were labeled using Hoechst33268 (Sigma-Aldrich). Following staining, embryos were placed in Mowiol® mounting medium (Calbiochem Merck; USA) on microscope glass slides and gently squashed under the coverslips before sealing. Images were acquired using a Leica DMR fluorescence microscope (Leica, Germany) and a Leica DFC-340FX camera system (Leica) and analyzed using Fiji software (National Institutes of Health, USA). For each embryo, total cell number was determined as the number of Hoechst stained nuclei, whereas the number of TE cells was determined by the number of CDX2 stained nuclei. The number of ICM cells was calculated by the difference between total and TE cell numbers.

3.2.3.5. Gene transcription analysis

Quantification of transcripts for Notch receptors (*Notch1-4*), Notch ligands (*Dll1*, *Dll4*, *Jagged1-2*), Notch effectors (*Hes1-2*), Wnt ligand *Wnt3a*, Wnt co-receptor *Lrp6* and pluripotency and differentiation marker genes (*Sox2*, *Oct4*, *Klf4* and *Cdx2*) was performed by quantitative real-time PCR (qRT-PCR) on individual 3.5 dpc embryos using the primers presented in Annex I. *Rps29* and *Hprt1* were used as housekeeping genes after establishing their stability between developmental stages and treatment groups (data not shown). RNA extraction from single embryos was performed with Arcturus® PicoPure™ RNA Isolation Kit (Applied Biosystems, ThermoFisher Scientific) following manufacturer's instructions and DNA digestion with RNase-free DNase Set (Qiagen, Germany). RNA concentration and purity were assessed spectrophotometrically at 260 and 280nm (NanoDrop™ 2000c, ThermoFisher Scientific). Complimentary DNA (cDNA) synthesis was performed using Maxima First Strand cDNA Synthesis Kit for qRT-PCR (ThermoFisher Scientific) using 20 ng of total RNA in each reaction. cDNA pre-amplification was achieved with SSoAdvanced™ PreAmp Supermix (BioRad, CA, USA) using a primer pool for genes including *Notch1-4*, *Dll1* and *4*, *Jagged1-2*, *Hes1-2*, *Wnt3a*, *Lrp6*, *Sox2*, *Oct4*, *Klf4*, *Cdx2* and housekeeping genes *Rps29* and *Hprt1*. Pre-amplified cDNA was diluted 1:10 in Tris-EDTA buffer and kept at -20°C until qRT-PCR analysis. qRT-PCR was performed in duplicate wells in StepOne Plus™ (Applied Biosystems), in 96-well optical reaction plates (Applied Biosystems) using the universal temperature cycles: 10 min of pre-incubation at 95 ^{o}C, followed by 40 two-temperature cycles (15 s at 95 ^{o}C and 1 min at 60 ^{o}C). Melting curves were acquired for every reaction plate to ensure that only wells with a single melting curve peak were analyzed. Each reaction used 10 µL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 2 µL of diluted pre-amplified cDNA (0.2 ng cDNA) and 80 nM of each primer in a total reaction

volume of 20 µL. The specificity of the obtained PCR products was confirmed by DNA sequencing. The data of relative mRNA quantification was analyzed with the comparative Ct method, using the average of *Rps29* and *Hprt1* as endogenous housekeeping genes. Treated embryos (DAPT, DKK1 and DAPT+DKK1 groups) were compared to untreated embryos (Control group as calibrator), and 5-7 embryos of each developmental stage (CM, BL, EBL) were evaluated within each group. Also, to evaluate gene transcription along development, BL and EBL were compared to CM (as calibrator).

3.2.3.6. Gene expression analysis - immunocytochemistry

Embryos (3.5 dpc BL, n=5; and EBL, n=5) were fixated, permeabilized and unspecific antigen blocked as described above. Incubation with primary antibodies, diluted in blocking solution, was done overnight at 4^{0}C. Primary antibodies were diluted as follows: rabbit anti-N1ICD (ab8925, Abcam, Cambridge, UK) diluted 1:100 and rabbit anti-HES1 (ab71559, Abcam) diluted 1:100. Negative IgG controls were performed using rabbit IgG (ab27478, Abcam) at the appropriate dilutions. Embryos were washed in PBS (4 × 5 min) and incubated with AlexaFluor® 594 chicken anti-rabbit (A11012, Life Technologies, USA) secondary antibody diluted 1:300 in blocking solution, for 30 min, at room temperature. Embryos were then washed 2 × 10 min in PBS followed by Hoechst33268 (Sigma-Aldrich) nuclear labeling and finally mounted in ProLong™ Gold Antifade Mountant (Life Technologies). Images were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with an optical magnification of 400× and treated with Fiji software.

3.2.3.7. Embryo transfer and pregnancy evaluation

Embryos were transferred to 2-3 month old pseudo pregnant CD1 recipient females. Pseudopregancy was induced by housing the females with vasectomized males overnight. Only females with a visually confirmed vaginal plug the following morning were used. Three days after pseudopregnancy induction (1 day asynchrony), 13-14 morphologically normal 4.0 dpc BL and EBL were transferred to each recipient (6-7 embryos into each uterine horn) according to standard protocols (Nagy et al. 2003). Following embryo transfer, recipients were euthanized either at day 5 of gestation (Day5; 48 hours after embryo transfer), or at day 18 of gestation (Day18; 16 days after embryo transfer).

At Day5, the detection of implantation sites was performed through the intra venous injection of 1% (w/v) Evans blue dye (Sigma-Aldrich) in the caudal vein of the dam. The

injection was performed under general anesthesia and the dye was allowed to perfuse the tissues for 5 min before euthanasia (performed as in embryo donors). The uterus was then exposed and the number of implantation sites recorded. At Day18, following euthanasia of the dam, the whole uterus was submerged in ice cold water for 15 minutes to euthanize the fetuses (American Veterinary Medical Association Panel on Euthanasia 2013). The number of fetuses and resorption sites and the weight of each fetus were recorded.

3.2.3.8. Fetal sexing

Fetal sexing was performed according to the protocol described by Clapcote and Roder (2005). Briefly, purified DNA was obtained from the tails of individual fetuses, and kept at -20^{0}C until analysis. Each PCR reaction was performed using 2 µL of purified DNA solution, 0.5 µM of each primer (Fwd: 5'-CTGAAGCTTTTGGCTTTGAG-3'; Rev: 5′-CCACTGCCAAATTCTTTGG-3′), 2 mM MgCl2, 0.4 mM dNTP, and 2U Taq enzyme (DFS-Taq DNA Polymerase, Bioron, Germany) in a final volume of 25 µL using the following cycling protocol: denaturation at 94^{0}C for 5 min, followed by 35 cycles of denaturation at 94^{0}C for 20 sec, annealing at 54^{0}C for 60 sec and extension at 72^{0}C for 40 sec, followed by a final extension step at 72^{0}C for 10 min. PCR products were visualized following electrophoresis in a 3% agarose gel and ethidium bromide staining. A single band (331bp) was considered of a female genotype and a double band (331bp + 302bp) was considered of a male genotype.

3.2.3.9. Statistical Analysis

Statistical analyses were performed using the statistical software Statistica7® (Statsoft, Tulsa, OK, USA, 2004). The chi-square test was used to evaluate treatment effects on *in vitro* cultured embryo survival and developmental kinetics, and on implantation rates after embryo transfer. Real-time RT-PCR data (Δ-Ct values) did not follow normal distribution and were transformed to Log x + 1 for normalization to allow the use of the subsequent parametric tests. Differential cell count and normalized Δ-Ct values were analyzed using a factorial ANOVA test, considering a 3 x 4 factorial design (3 developmental stages x 4 treatment groups). The weight of the fetuses was analyzed by ANCOVA, using the number of developed fetuses in each uterus as covariate. Significant results of the ANOVA and ANCOVA tests were further investigated *post-hoc* using the LSD test. Results were considered significant if $p < 0.05$.

3.2.4. Results

3.2.4.1. Embryo survival and developmental kinetics

Table 7 shows the combined data of the 27 *in vitro* culture sessions for embryo survival rate (at 3.5 dpc, 4.0 dpc and 4.5 dpc). The total number of embryos evaluated at each time-point is different due to the temporal permanence in culture of embryos allocated to different evaluations. Compared to group Control, single blockades (Notch or Wnt) had no effect on embryo survival rate, whereas the double blockade decreased embryo survival at 4.0 dpc and 4.5 dpc.

Table 7: Effect of Notch or/and Wnt blockade on mouse embryo *in vitro* survival rate.

	Culture time-point					
	3.5dpc		4.0dpc		4.5dpc	
Group	Total n	Viable n (%)	Total n	Viable n (%)	Total n	Viable n (%)
Control	620	534 (86)	329	294 (89) ^{a}	117	103 (88) ^{cd*}
DAPT	433	371 (86)	280	239 (85) ^{ab}	96	85 (89) ^{cd}
DKK1	447	385 (86)	193	174 (90) ^{a}	82	74 (90) ^{c}
DAPT+DKK1	390	323 (83)	205	166 (81) ^{b}	81	63 (78) ^{d*}

Effect of Notch or/and Wnt blockade (respectively DAPT, DKK1 and DAPT + DKK1), on mouse embryo *in vitro* survival rate (% of non-degenerated morphologically normal embryos) at 3.5 dpc (after *in vitro* culture for 24 hours), 4.0 dpc (36 hours) and 4.5 dpc (48 hours). Combined data of 27 *in vitro* culture sessions (6 ending at 4.5 dpc, 11 ending at 4.0 dpc and 10 ending at 3.5 dpc). Values with different superscripts differ significantly: a b, $p < 0.01$; c d, $p < 0.05$; * $p = 0.05$.

Figure 10 shows the effect of Notch and/or Wnt signaling blockades on embryo developmental kinetics. Compared to group Control, Notch blockade slowed development at 3.5 dpc (increasing the CM rate while decreasing the EBL rate) and at 4.0 dpc (increasing the BL rate while decreasing the HBL rate), but had no further effect on embryo kinetics at 4.5 dpc. In contrast, Wnt blockade fastened development at 4.0 dpc (decreasing the BL rate while increasing the EBL rate). The double blockade slowed development at 3.5 dpc (increasing the CM rate while decreasing the EBL rate), 4.0 dpc (increasing the BL rate while decreasing the HBL rate) and 4.5 dpc (increasing the EBL rate while decreasing the HBL rate) in a more expressive manner than the Notch blockade alone. The opposing effects of Notch and Wnt blockades, compared to each other, were evident at the three time-points.

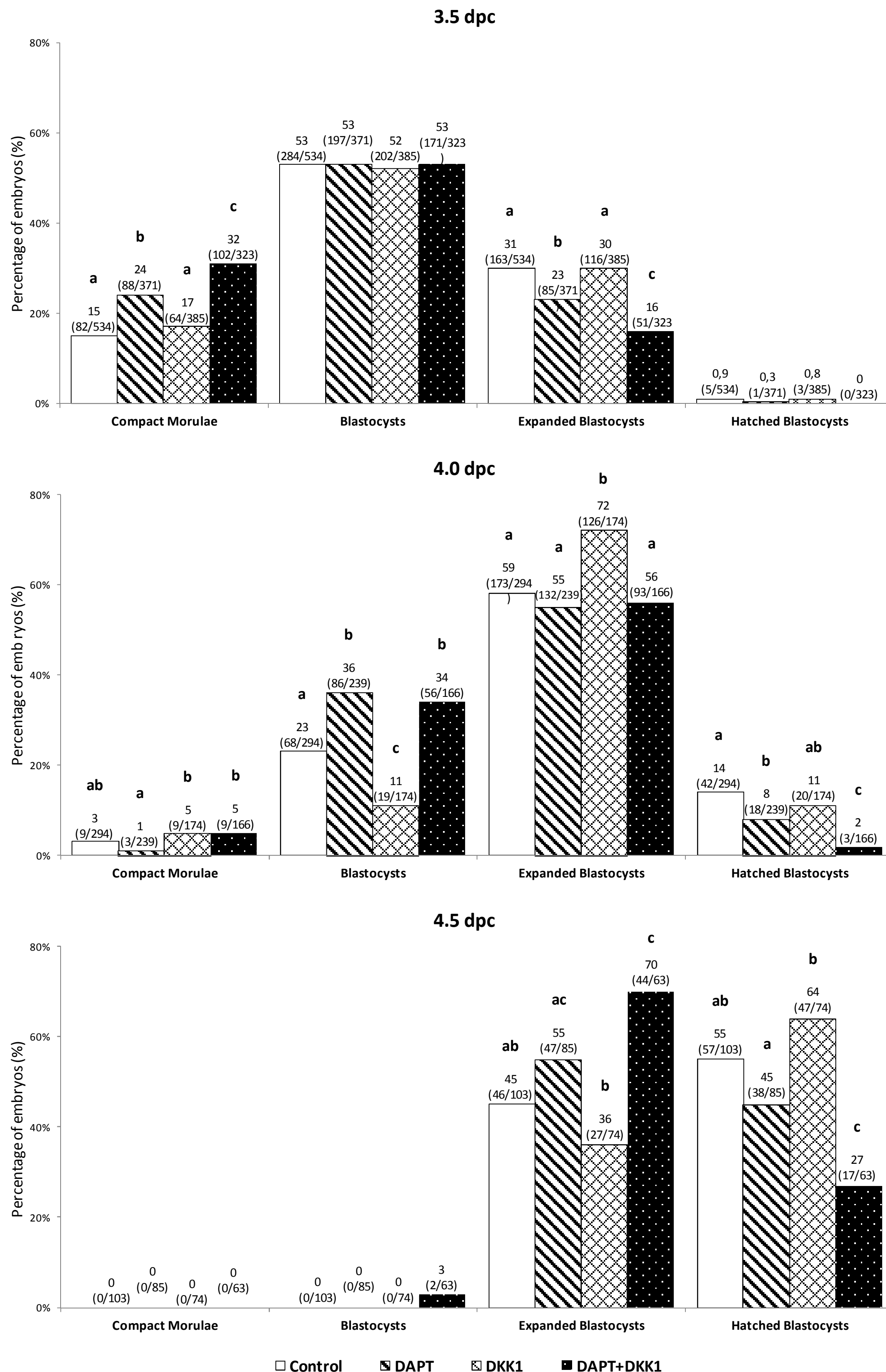


Figure 10: Effect of Notch, Wnt and double blockades on *in vitro* mouse embryo developmental kinetics.

Effect of Notch (DAPT), Wnt (DKK1) and double (DAPT+DKK1) blockades on mouse embryo developmental kinetics. Bars represent percentage of each developmental stage in each treatment group. Combined data of 27 *in vitro* culture sessions (6 ending at 4.5 dpc, 11 ending at 4.0 dpc and 10 ending at 3.5 dpc). Within each developmental stage, bars with different letters differ significantly, $p < 0.05$.

3.2.4.2. Embryo differential cell count

Figure 11 and Figure 12 show the effects of blockades on ICM, TE and total cell counts, and on ICM:TE ratio. Compared to group Control, Notch and Wnt blockades had no effect on Total, TE and ICM cell number (although DAPT tended to decrease ICM cell number; p = 0.08), whereas the double blockade decreased TE and total cell number in CM. Notch blockade decreased the ICM:TE ratio in CM and BL.

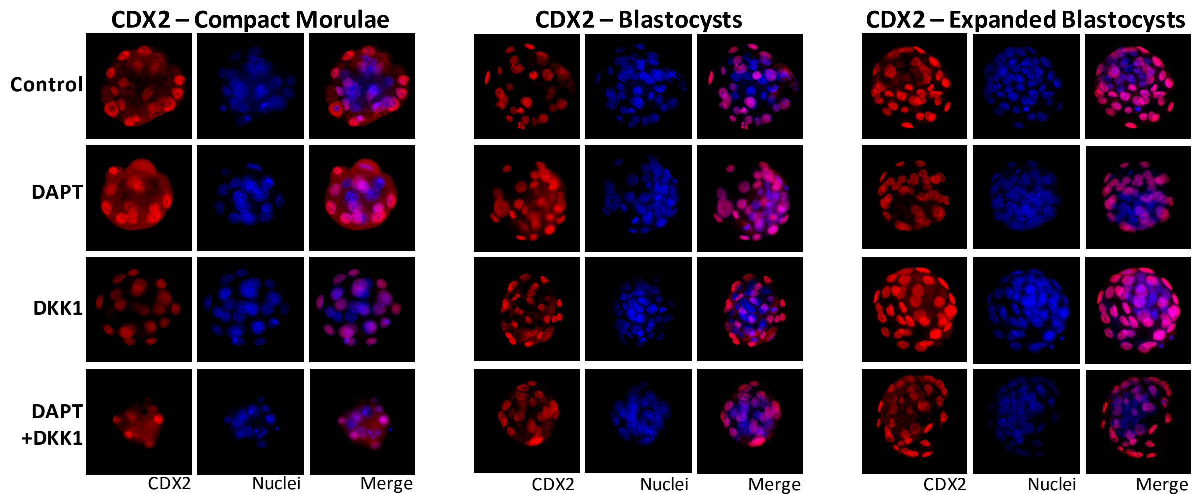


Figure 11: Immunostaining of CDX2 in 3.5 dpc mouse embryos

Immunostaining of CDX2 in 3.5dpc embryos in groups Control, Notch blockade (DAPT), Wnt blockade (DKK1) and Double blockade (DAPT+DKK1). CDX2 positive nuclei are stained red and all nuclei are stained blue (Hoechst). Magnification 400×.

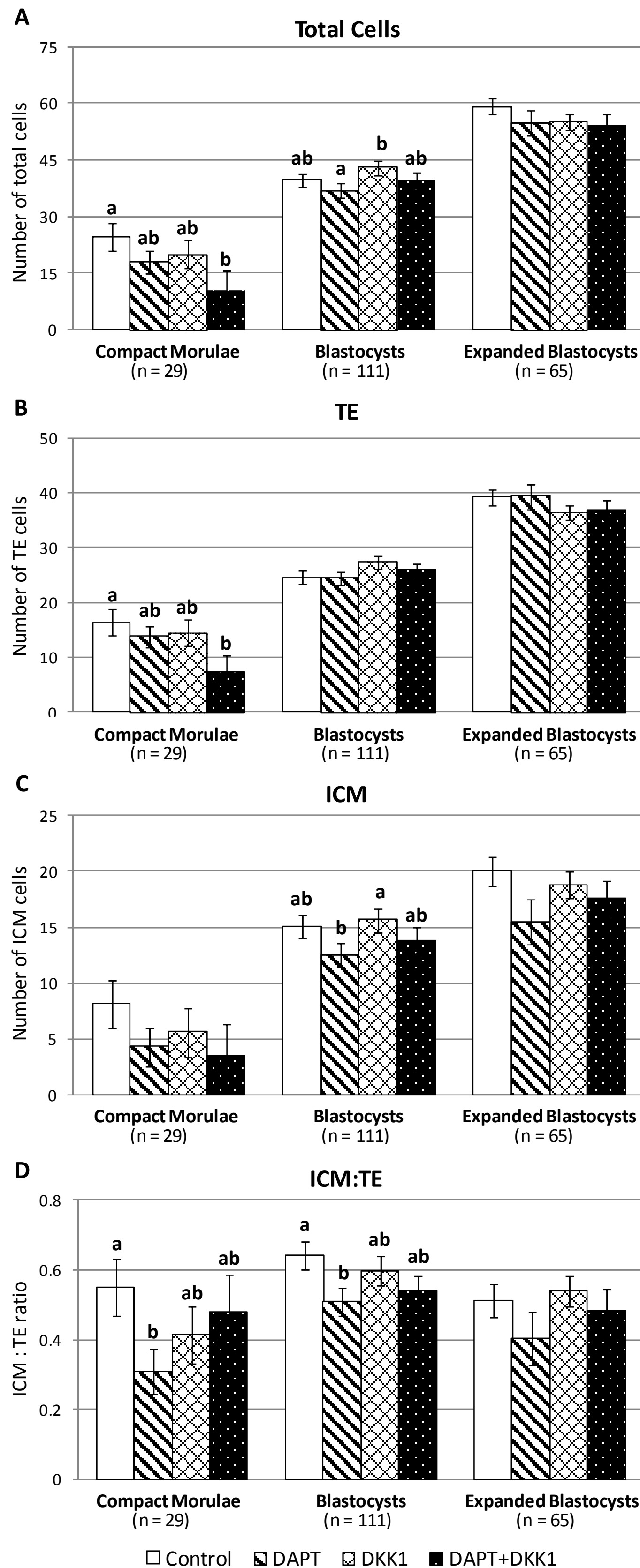


Figure 12: Effects of Notch, Wnt and double blockade on 3.5 dpc embryo differential cell count

Effects of DAPT (Notch blockade), DKK1 (Wnt blockade) and DAPT+DKK1 (double blockade) on 3.5 dpc embryo total (A), trophectoderm (TE) (B) and inner cell mass (ICM) (C) cell number, and ICM:TE ratio (D). Bars represent the mean values and error bars the s.e.m. Within the same developmental stage, bars with different letters differ significantly, $p < 0.05$.

3.2.4.3. Transcription of pluripotency and differentiation marker genes

Sox2, *Oct4*, *Klf4* and *Cdx2* transcripts were present in all evaluated embryos. Figure 13 illustrates the profile of relative transcription (relative fold change to CM as the calibrator) of these genes along development to BL and EBL. As expected, embryos from the Control group showed a significant increase in transcription of *Sox2* at the BL stage, followed by a significant decrease at the EBL stage, although at this latter stage still significantly higher than at the CM stage. This pattern was disrupted by all blockades, as embryos from these groups showed no variation in transcription of *Sox2* from CM to EBL (Figure 13A). This transcription analysis is further clarified in Figure 14A, which illustrates the relative transcription (relative fold change) to Control group mean (as a calibrator). As shown, the three blockades significantly increased transcription levels of *Sox2* (about 4 folds) in CM, compared to Control group, *i.e.* the physiological increase in *Sox2* transcription was anticipated. In contrast, Notch blockade significantly decreased *Sox2* transcription levels at the BL stage (Figure 14A).

The profile of relative transcription of *Oct4* in Control group embryos was constant between the CM and BL stages, followed by a significant increase at the EBL stage (Figure 13 B; Figure 14 B), as observed by others (reviewed by Wu and Schöler 2014). This pattern was not changed in Notch or/and Wnt blockades. The relative transcription of *Klf4* in Control group embryos was constant between the CM and BL stages, followed by a significant decrease at the EBL stage (Figure 13C; Figure 14C). Notch blockade changed this pattern by significantly decreasing transcription levels of *Klf4* in BL, and significantly increasing levels in EBL. Wnt and double blockades also changed the pattern of transcription levels of *Klf4* by stabilizing it from the CM to the EBL stage. In control embryos, *Cdx2* transcription was constant from the CM to the ExpBL stages. This pattern was disrupted in Notch and double blockade embryos (Figure 13D; Figure 14D), where a significant increase in transcription levels of *Cdx2* was observed at the EBL stage, although this effect was already noticeable at the BL stage in double blockade embryos.

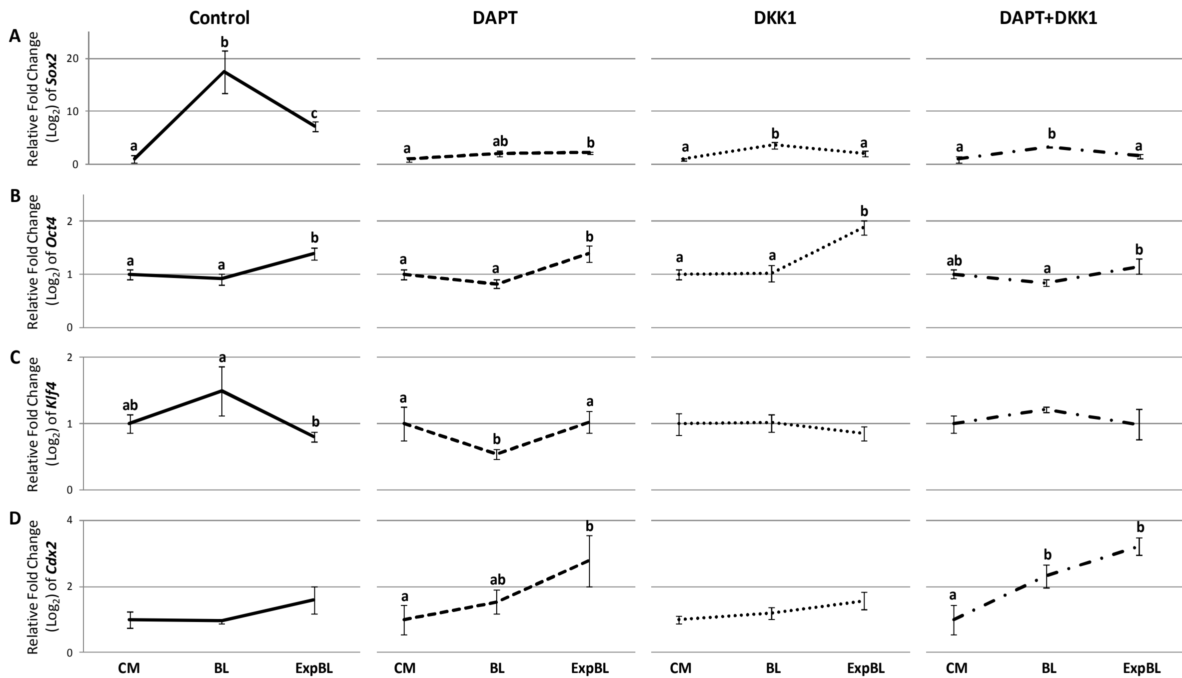


Figure 13: Effect of Notch, Wnt and double blockades on the profile of *Sox2*, *Oct4*, *Klf4* and *Cdx2* transcription throughout development

Profiles of relative transcription levels of *Sox2* (A), *Oct4* (B), *Klf4* (C) and *Cdx2* (D) in untreated control or following treatment with DAPT (Notch blockade), DKK1 (Wnt blockade) and DAPT+DKK1 (Double blockade) 3.5 dpc embryos. Lines represent folds of transcription relative to compact morulae (calibrator) levels ± s.e.m. (n = 6-7 embryos per each developmental stage and treatment group). For each line, points with different letters differ significantly, $p < 0.05$.

Figure 14: Effect of Notch, Wnt and double blockades on relative transcription of *Sox2*, *Oct4*, *Klf4* and *Cdx2* in 3.5 dpc embryos

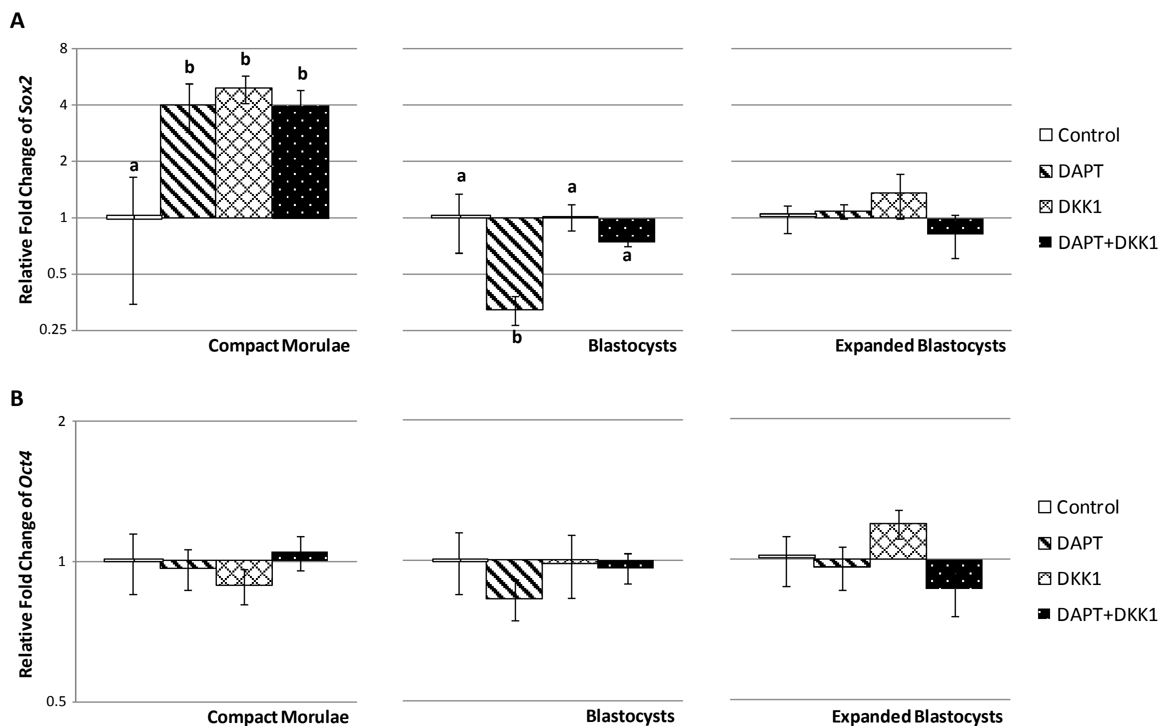
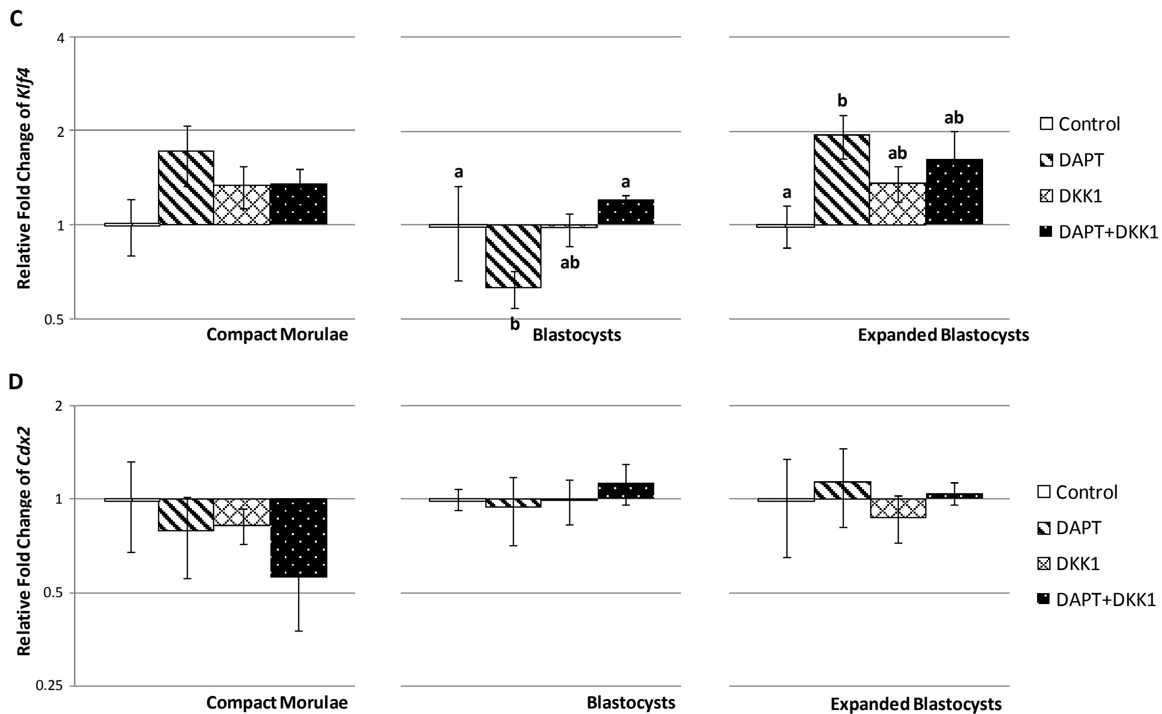


Figure 14 (continuation)



Relative transcription of *Sox2* (A), *Oct4* (B), *Klf4* (C) and *Cdx2* (D) in 3.5dpc embryos of groups Control (calibrator), Notch blockade (DAPT), Wnt blockade (DKK1) and double blockade (DAPT+DKK1). Bars represent folds of transcription relative to control levels ± s.e.m. (n = 6-7 embryos per each developmental stage and treatment group). Within each developmental stage, bars with different letters differ significantly, a, b, c: $p < 0.05$.

3.2.4.4. Transcription of Notch and Wnt genes

Detection of transcripts of Notch components in Control group embryos was in accordance with a previous publication (Batista et al. 2020). Ligand *Jagged1* transcription was significantly affected by Notch and double blockades (Figure 15): Notch blockade decreased *Jagged1* transcription at the EBL stage, whereas the double blockade decreased transcription at the BL stage (also compared to single blockades). Effector *Hes1* transcription was significantly decreased by Wnt and double blockades at the EBL stage (Figure 15B), and Notch had an opposite effect to Wnt and double blockades at the CM stage. Regarding the Wnt pathway components, treatments had no effect on *Wnt3a* transcription, but *Lrp6* transcription was significantly increased by Wnt blockade at the CM and BL stages, whereas at the EBL stage was significantly decreased by Notch blockade (Figure 15C). Notch and Wnt blockades induced opposite effects in *Lrp6* transcription at all development stages.

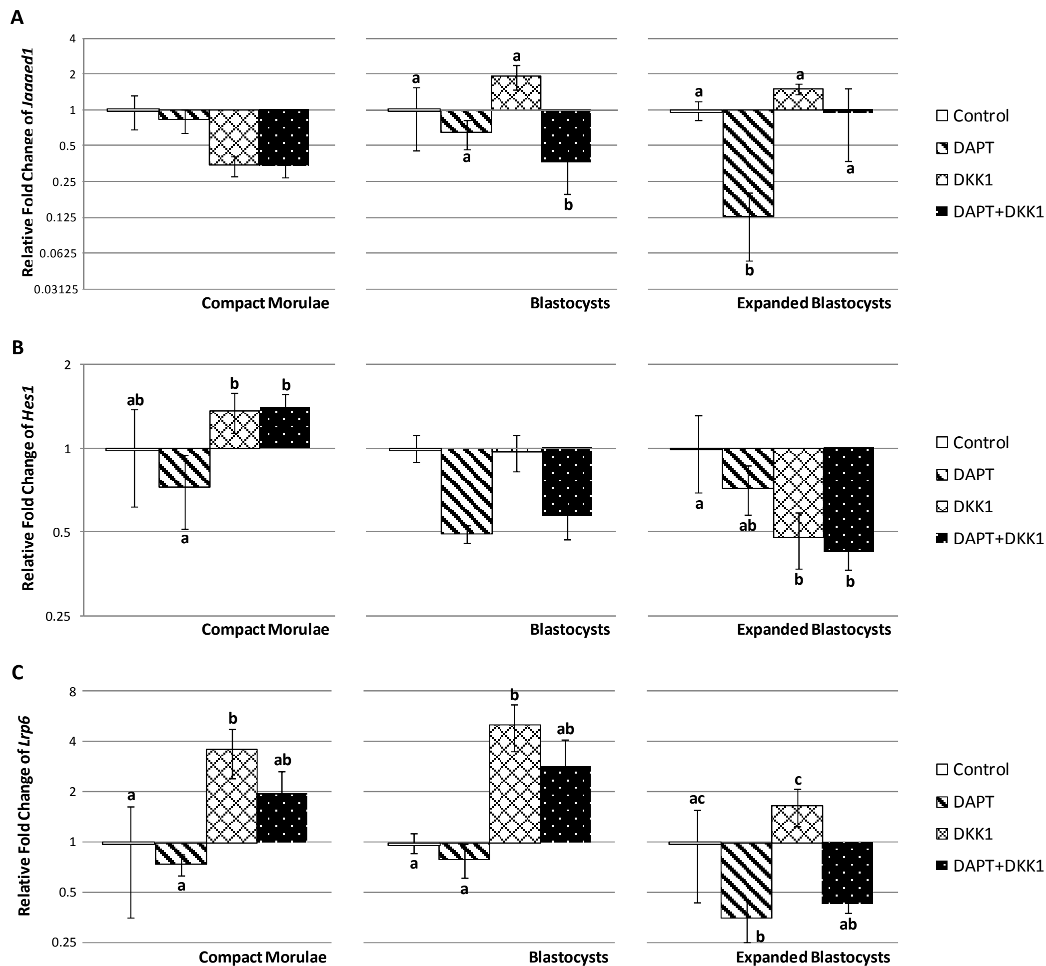


Figure 15: Effect of Notch, Wnt and double blockades on relative transcription of *Jagged1*, *Hes1* and *Lrp6* in 3.5 dpc embryos

Relative transcription of *Jagged1* (A), *Hes1* (B) and *Lrp6* (C) in 3.5 dpc embryos of groups Control, Notch blockade (DAPT), Wnt blockade (DKK1) and Double blockade (DAPT+DKK1). Bars represent folds of transcription relative to control (calibrator) levels ± s.e.m. Within each developmental stage, different letters differ significantly, a,b,c: $p < 0.05$.

3.2.4.5. Gene expression

Canonical Notch signaling activation was confirmed by nuclear detection of N1ICD in Control group embryos (Figure 16A). As expected, Notch blockade decreased N1ICD nuclear expression (Figure 16A). However, this was also accomplished by the Wnt blockade and in a more obvious way by the double blockade. Only embryos from the Wnt blockade group showed an ectopic N1ICD nuclear location in ICM cells. In accordance with the team´s previous study (Batista et al. 2020), HES1 was detected in the nucleus of Control group embryos, as well as in a diffuse cytoplasmic pattern (Figure 16B). Strong nuclear localization and absence of cytoplasmic staining of this effector was observed following the Wnt blockade and, in a more evident way, in the double blockade.

Figure 16: Effect of Notch, Wnt and double blockades on N1ICD and HES1 immunostaining in 3.5 dpc embryos

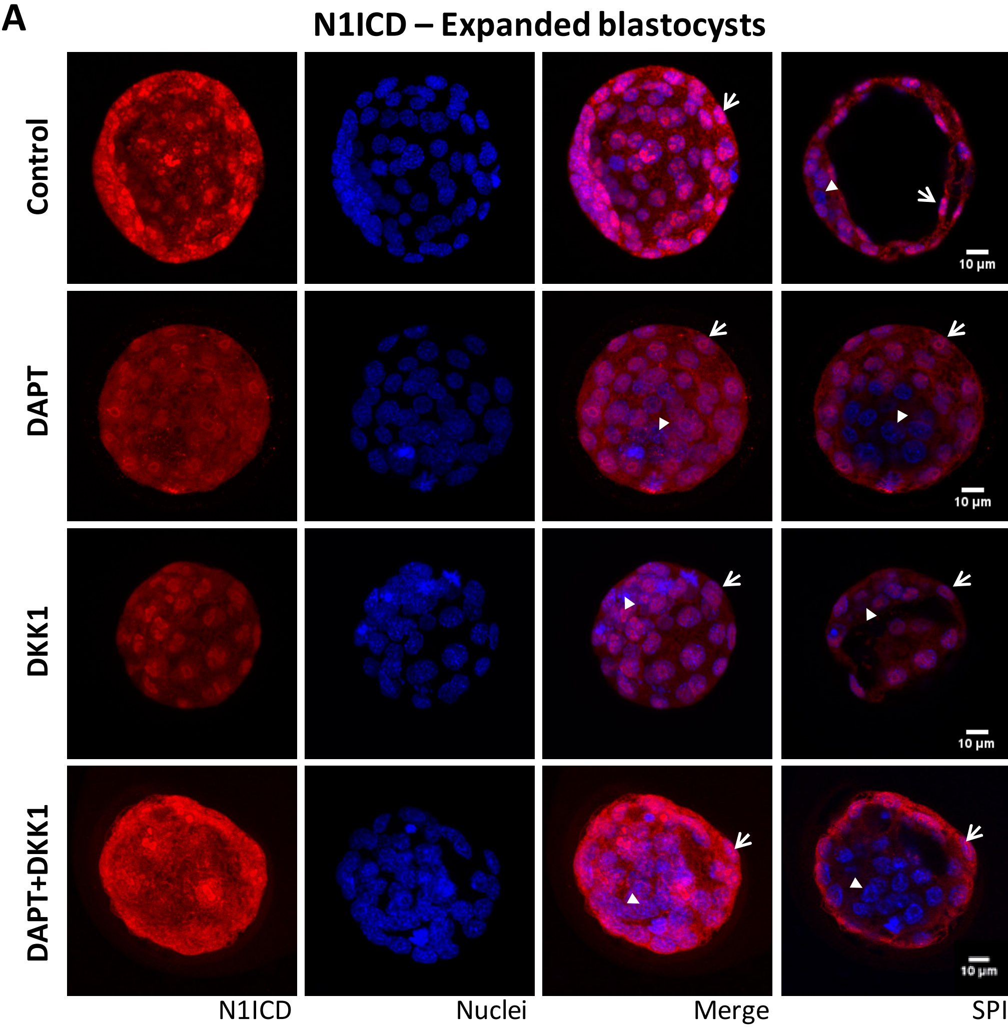
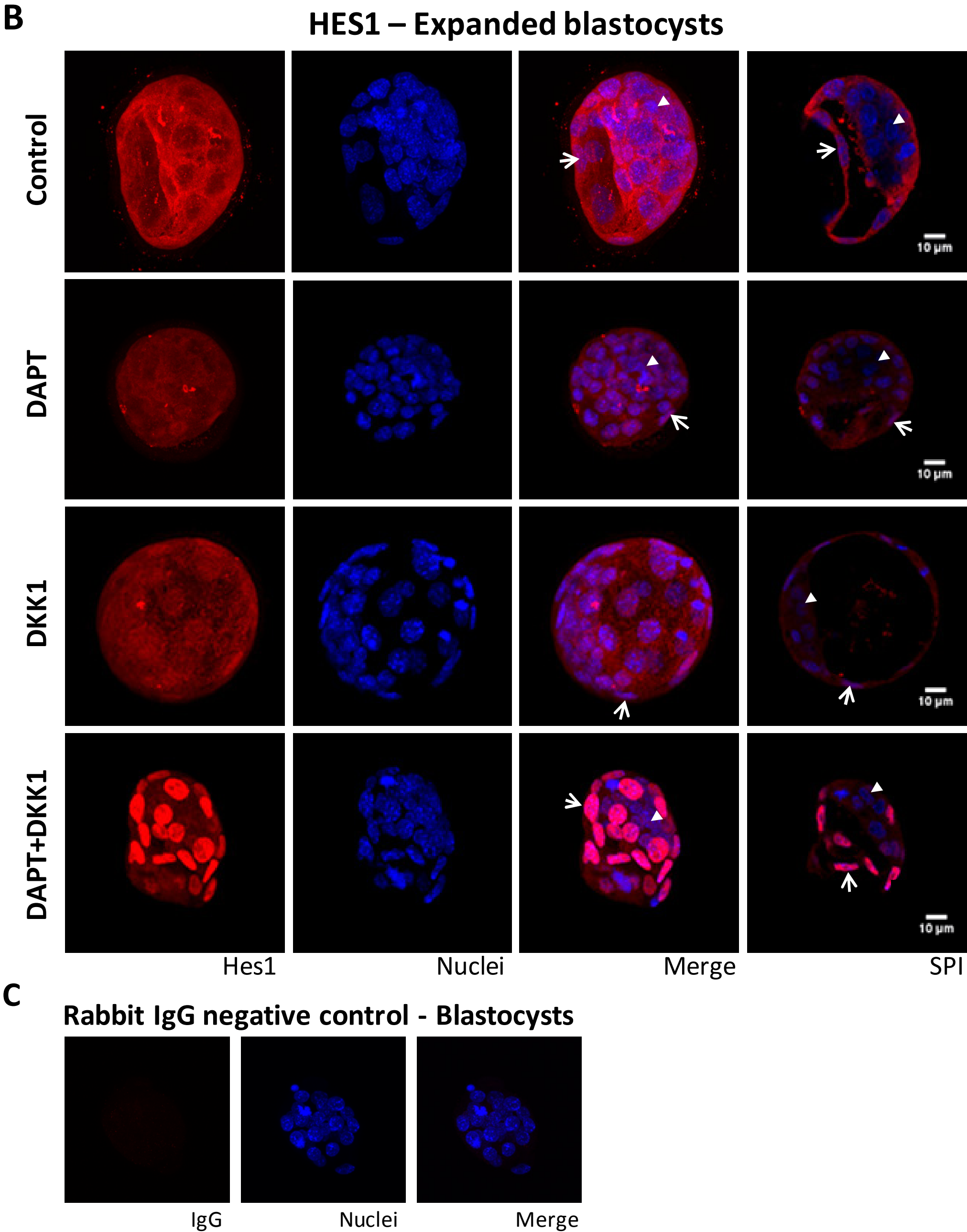


Figure 16 (continuation)



Immunostaining of N1ICD (A) and HES1 (B) and IgG negative control (C) in 3.5 dpc embryos in Control, Notch blockade (DAPT), Wnt blockade (DKK1) and Double blockade (DAPT+DKK1) groups. Target proteins are stained red and nuclei are stained blue (Hoechst). Images in the first three columns are maximum intensity projections of the obtained Z-stack; the fourth column is a representative single plane image (SPI). Arrows (→) indicate presumptive trophectoderm cells and arrow heads (►) indicate presumptive inner cell mass cells. Scale bar 10 µm.

3.2.4.6. Implantation and fetal development

Implantation rate at Day5 was not affected by blockades (Figure 17A). However, at Day18, the rate of fully developed fetuses was significantly decreased by Notch and double blockades, the latter inducing a significantly more pronounced effect than the former (Figure 17B). The sex rate of Day18 fetuses was not affected by Notch and double blockades, but Wnt blockade tended to increase the male rate in fully developed fetuses (Control: 49% vs. DKK1: 68%, $p = 0.05$). Fetal sex had no effect on fetal weight (data not shown). However, Day18 fetal weight was significantly affected by blockades, with Notch and Wnt blockades inducing opposing effects (Figure 18): Notch blockade decreased fetal weight, whereas Wnt blockade increased fetal weight.

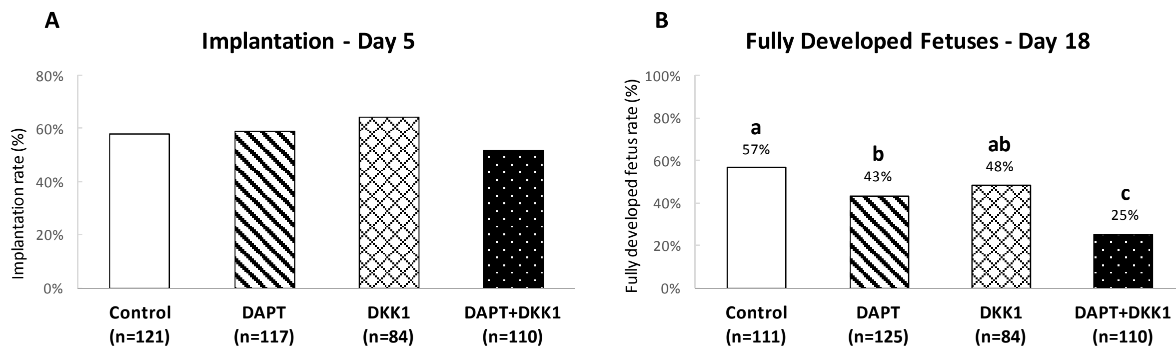


Figure 17: Effect of Notch, Wnt and double blockades on implantation rate at Day5 and viable fetal rate at Day18 of gestation

Effect of Notch (DAPT), Wnt (DKK1) and double (DAPT+DKK1) blockades on (A) implantation rate at Day5 of pregnancy and (B) viable fetal rate at Day18 of pregnancy. Bars with different letters differ significantly: abc, $p < 0.05$

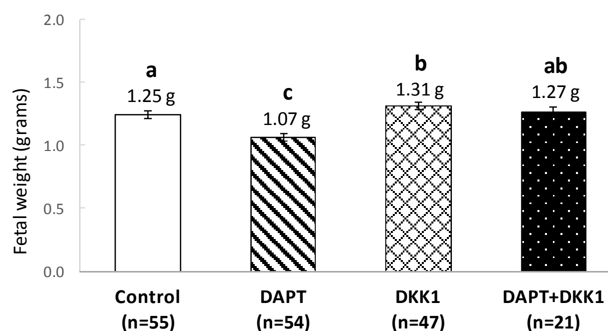


Figure 18: Effect of Notch, Wnt and double blockades on mouse fetal weight at Day18 of pregnancy

Effect of Notch (DAPT), Wnt (DKK1) and double (DAPT+DKK1) blockades on Day18 of pregnancy mouse fetal weight. Bars represent adjusted means for the number of fetuses in each litter ± s.e.m. Bars with different letters differ significantly: a,b,c $p < 0.05$.

3.2.5. Discussion

This study is the first to evaluate the interplay between Notch and Wnt cell signaling pathways in early embryo development and the role of this early signaling on subsequent fetal development until term. Results evidence that Notch and Wnt signaling regulate the pace of developmental kinetics and the first embryonic cellular differentiation, which affect embryo development and future fetal development until term. This highlights the existence of a carry-over effect of these signaling pathways from the first embryonic cell fate assignment to events occurring at fetal development stages, influencing fetal weight and survival rate.

The study confirmed the individual embryonic transcription and expression of Notch components, and the active signaling status of the Notch canonical pathway (Menchero et al. 2019; Batista et al. 2020). The blockade of canonical Notch signaling was confirmed by the downregulation of transcription of *Jagged1* and decrease in N1ICD nuclear staining, as seen in other scenarios (Reznikova et al. 2009; Hu et al. 2018), whereas the blockade of canonical Wnt signaling was confirmed by the upregulation of transcription of Wnt co-receptor *Lrp6*, as also observed by others (Khan et al. 2007; Li et al. 2010). Therefore, the phenotypes here reported are elucidative of the physiological role of both pathways in embryonic development and Figure 19 shows a schematic representation of these major actions of Notch and Wnt signaling pathways on early embryo development. However, as canonical signaling might have not been fully abolished and non-canonical signaling cannot be excluded, the observed phenotypes might only reflect a part of the role of these signaling pathways in early development.

Precise embryo developmental kinetics is essential for embryo-maternal interaction within the temporally limited window of implantation (reviewed by Carson et al. 2000). Results from this study indicate that Notch and Wnt are involved in the regulation of the pace of embryo kinetics in an opposing manner: Notch blockade slows development, indicating that, physiologically, Notch stimulates progress in development; in contrast, Wnt blockade fastens development, indicating that, physiologically, Wnt halts progress in development. However, this latter effect of slowing development is precisely timed, as the effect of blocking Wnt occurs at 4.0 dpc, at the time of blastocyst expansion and when embryos have arrived at the uterus (Yoshinaga 2013), having no further effect on the hatching rate. The endometrium produces DKK1 during the window of implantation in several species, including mice (Kao et al. 2002, Li et al. 2008), and this production has been associated with increased fertility in cows (Minten et al. 2013). Alltogether, the present and above studies indicate that embryonic Wnt canonical signaling must be downregulated during the implantation window, allowing faster embryo development towards implantation. However, the balanced action of Notch

and Wnt is necessary for physiological pace of development. In fact, the double blockade of Notch and Wnt significantly exacerbated the effects of Notch blockade, slowing development and hindering hatching. This may indicate a mechanism by which Wnt may partially compensate Notch in its absence.

The immunostaining for CDX2 showed that Notch signaling regulates the ICM:TE ratio in CM and BL, as Notch blockade decreased this ratio, most likely due to a decrease in ICM cells. This phenotype was also observed in double blockade embryos, possibly resulting from the blockade of the Notch pathway. CDX2 is a transcriptional regulator essential for the segregation of ICM and TE lineages at the BL stage (Strumpf et al. 2005), and Notch signaling was implicated as a direct positive regulator of CDX2 expression in early embryo development (Rayon et al. 2014; Watanabe et al. 2017; Menchero et al. 2019). In their study, Rayon et al. (2014), used RBPJ knockout embryos and a γ-secretase inhibitor, and observed that proper CDX2 expression and TE lineage specification was regulated by the cooperation between the Notch and Hippo pathways. Additionally, Wnt was implicated as a regulator of cell number and of TE differentiation in pig (Lim et al. 2013) and bovine blastocysts (Denicol et al. 2013). The here observed effect on ICM, rather than on TE cells, contrasts with data from Rayon et al. (2014), but is in accordance to what was observed in human embryonic stem cells, where Notch inhibition decreased cellular proliferation, although not affecting its differentiation (Fox et al. 2008). The inconsistency between this study and that of Rayon et al. (2014) may rely on the moment of Notch blockade, as in the latter study the blockade was before the stochastic patterning of blastomeres at the CM stage. This is in line with the biphasic and dual role for Notch, initially at the very beginning of TE formation and later regulating ICM proliferation/ maintenance, as described by Menchero et al. (2019).

The blockade of either Notch or Wnt, strongly stimulated the transcription of *Sox2* in CM, which is expressed in all blastomeres at this stage (Keramari et al. 2010). This rise in transcription precedes the physiological rise observed in Control group BL, which indicates that both Notch and Wnt are repressing transcription of *Sox2* in CM, until cell fate commitment is fully established at this stage. In BL, the physiological rise in *Sox2* transcription follows the increase in cell number (Wang et al. 2004; Keramari et al. 2010). However, Notch is necessary for *Sox2* transcription in BL, since Notch blockade decreased *Sox2* transcription at this stage, as previously described in other scenarios (Neves et al. 2011; Batchuluun et al. 2017; Batista et al. 2020).

Correct expression of OCT4 is required for the control of stem cell differentiation, as OCT4 deregulation can cause cellular differentiation (Niwa et al. 2000). The Wnt and double blockades changed the pattern of *Oct4* transcription from CM to EBL. Since SOX2 and OCT4

are key regulators of *Nanog* transcription (Rodda et al. 2005), and thus maintenance of the ICM pluripotent state, the disruption of the available amounts of these transcription factors may affect ICM cell pluripotency. Directly interacting with SOX2 and OCT4, KLF4 is required to maintain self-renewal in ES cells (Wei et al. 2009). The physiological *Klf4* transcription profile, as observed in Control group embryos, was significantly affected by the Notch blockade, which decreased transcription in BL and increased it in EBL. Therefore, physiologically, Notch is a key stimulator of *Klf4* transcription at blastulation, and by the time blastocysts have matured acts as a transcription repressor. However, as the double blockade reverses the effect of Notch blockade on blastocyst *Klf4* transcription, this may indicate that the Notch stimulatory effect is mediated by Wnt.

Notch and Wnt pathways are known to interplay using several strategies including the co-operative regulation of transcription targets, the induction or inhibition of the production of each other pathway components, and the use of the components of one pathway by the other (Collu et al. 2014). As predicted, the blockade of Notch and Wnt pathways disrupted transcription of their own components. Additionally, the blockade of Notch also affected the transcription of Wnt components and vice versa. Specifically, Notch blockade downregulated *Lrp6* transcription and Wnt blockade downregulated *Hes1* transcription. This latter downregulation could be due to the non-physiological nuclear HES1 expression, which is known to act in a negative feedback regulatory mechanism on its own transcription (Hirata et al. 2002). The double blockade induced effects different than the cumulative effects of Notch and Wnt blockades alone, as seen in the downregulation of *Jagged1* transcription in BL. These results indicate that the phenotypes induced by Notch and Wnt interplay are the result of both their own pathway activation and interference in the activation of each other. In general, both pathways act by repressing the other, keeping activation levels in a biphasic manner from the CM stage – where Notch needs to be activated, and the EBL stage – where Wnt needs to be silenced. The key regulators of Notch signaling interaction with Wnt seems to be the ligand JAGGED1 and effector HES1.

Although Notch and Wnt pathways were only blocked for a brief period of 36 hours, this had a major impact on later fetal development. The effects of blockades were not noticeable at Day5, indicating that embryos maintained the ability to develop until implantation. In contrast, the effects of blockades were evident at Day18. In fact, Notch blockade significantly decreased fetal development until term, which was exacerbated in the case of simultaneous Wnt blockade, also addressing the interplay between these two pathways. This clearly underlines two highly relevant reproductive biology issues: i) the major regulatory role of Notch and Wnt signaling pathways on early embryonic development; and ii)

the fact that early disruptions during the first cellular lineage commitment stage will have profound deleterious effects later on during fetal development until term (Noli et al. 2015, Almagor et al. 2016). The brief Notch blockade in early development not only hampered fetal survival to term, but also decreased fetal weight. Interestingly, this may be related to the observed decrease in ICM:TE ratio at first differentiation commitment, slowing development of ICM-derived cells. In contrast, and again reflecting an opposing role, the brief Wnt blockade increased fetal weight. A similar effect was observed in bovine embryos treated with DKK1 by Denicol et al. (2014), who reported that treated embryos tended to show increased length at day 34 of gestation. It would be of most scientific and medical interest to evaluate if this overgrowth is beneficial for the newborn health and if it is involved in the ART-derived large offspring syndrome described in several mammalian species.

In conclusion, this study provides experimental data evidencing that Notch and Wnt signaling pathways are active and interplaying at the first cellular differentiation stage of early embryonic development. This interplay exhibit a major regulatory role in the pace of development, as its blockade induces a disruption in ICM:TE ratio, the profiles of transcription of pluripotency and differentiation marker genes, and the kinetics of development. Balanced Notch and Wnt signaling, often in an opposing manner but also in a combined manner, is a key requisite for physiological early embryo development. Disruption of this balance, even for a very short period during ICM and TE commitment and specification, induces deleterious effects on later fetal development to term. Alltogether, results highlights the relevance of this early embryonic stage for subsequent development and of the role of Notch-Wnt signaling interplay on embryo-fetal physiological development. This prompts Notch and Wnt pathways as therapeutic targets in the scope of reproductive medicine.

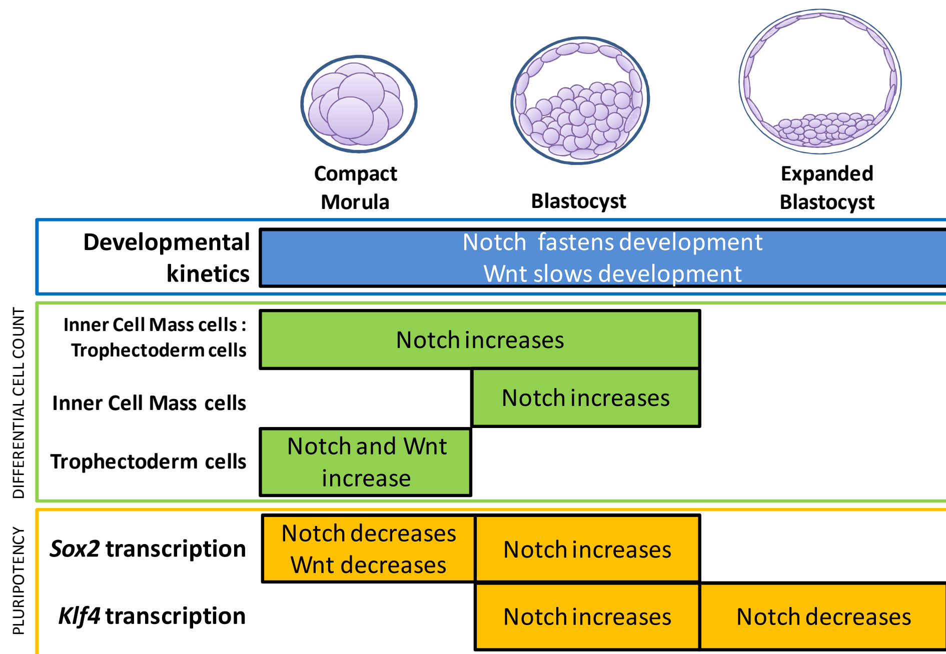


Figure 19: Schematic representation of the role of Notch and Wnt signaling on early embryo development.

4. DISCUSSION AND CONCLUSIONS

The work presented in this thesis confirms the activity of the Notch signaling pathway in early embryo development and further demonstrates its critical role at this stage. Results evidence that Notch signaling is implicated in the control of the first cell differentiation events and that its actions resonate beyond this stage, having consequences in the overall fetal development until term. Additionally, this work places Notch signaling as an active and pivotal player of the wider network of intercellular signaling pathways present in early embryos which includes the Wnt signaling pathway.

4.1. Notch signaling pathway in preimplantation mouse embryos

The earliest report of a systematic analysis of the presence of Notch components in preimplantation mouse embryos was performed in 2004, by Cormier and colleagues. To this effect, oocytes and staged embryos were obtained either by direct collection from superovulated females or by *in vitro* culturing these until the next developmental stage. Due to the limited amount of RNA an individual embryo (or oocyte) can yield, RNA isolation was performed from pools of 30-130 individuals and transcription analysis was performed by nested RT-PCR. This pool technique has the disadvantage of diluting eventual differences between individual embryos and giving a less accurate picture of transcript prevalence.

In the present work, transcription analysis was performed using the quantitative real-time RT-PCR (qRT-PCR) technique, which is a reliable tool to detect and measure minute amounts of nucleic acids in a wide range of biological samples (Bustin et al. 2009). Since an intercalating dye (SYBR® Green I) was chosen for the detection of amplification in detriment of labeled probes, because of its versatility and cost efficiency in detecting transcription of several genes (Kubista et al. 2006), further steps were taken to ensure the specificity of each reaction and avoid false positives, such as DNAse treatment and special primer design (flanking introns) (Peters et al. 2004). Additionally, only samples which produced a single melting curve peak specific to the target gene were quantitatively analyzed (Bustin et al. 2009). This method for analyzing transcription of individual preimplantation mouse embryos has clear advantages over the previously used technique for analysis of pools of embryos, since it is able to account for individual variability and can accurately determine if an individual embryo is transcribing a certain gene, quantify this transcription and relate this with the transcription of genes that control key cellular events at this stage, without being influenced by the averaging effect of the simultaneous analysis of several individuals in a pool.

In fact, we found some key differences from the results previously published (Cormier et al. 2004) regarding the prevalence of transcripts of Notch components at the several preimplantation stages. While both works found ubiquitous presence of *Notch1*, *Notch2*, *Jagged1* and *Jagged2* from CM to HBL stages, we could not confirm transcription of *Dll1* at any stage and found that *Notch3*, *Notch4* and *Dll4* transcription is not ubiquitous, but is rather embryo specific. These discrepancies may arise from embryo origin. In this work, both BL and HBL were obtained from 2.5dpc 8- and 16-cell embryos *in vitro* cultured until 3.5 dpc and 4.5 dpc, respectively, whereas Cormier and colleagues analyzed *in vivo* derived 3.5 dpc BL and 4.5 dpc HBL *in vitro* cultured for 24 hours. However, the most likely explanation arises from the different transcription analysis methodologies. Our methodology ensures the detection of very small amounts of mRNA copies from single embryos, highly reducing the possibility of obtaining false negative results. Additionally, the high accuracy of qRT-PCR (as well as the additional technical control confirmations) allows the exclusion of false positives resulting from unspecific amplifications of similar strands of nucleotides.

More recently, new transcription analysis techniques, such as single cell RNA sequencing (sc-RNAseq), have been developed with higher sensitivity and specificity, allowing an accurate analysis of very small amounts of biological material, such as a single blastomere (Guo et al. 2010; Tang et al. 2011; Deng et al. 2014). The evaluation of the transcriptome of a single cell gives us a detailed picture of regulatory relationships between genes within the same cell and can track the evolution of transcription patterns of cells of a given cell population across time. However, precise characterization of a given cell and its allocation to a given cellular population can prove somewhat challenging, since there are many potential states a cell can present as development or disease progresses. Although there are known markers for certain cell types, such as CDX2 in the case of TE cells in the early blastocyst, we can still observe great diversity between cell types (Hwang et al. 2018). In fact, analysis of individual blastomeres (Tang et al. 2011; Deng et al. 2014) show that the presence of these transcription markers is not an absolute sign of identity, and their relative quantity in the population, as well as their prevalence, needs to be taken into account. Additionally, the post transcriptional regulation of translation and biological activity is what better defines a given cell (Pujadas and Feinberg 2012; Harvey et al. 2017).

In these sc-RNAseq studies of single blastomeres, many components of the Notch signaling pathway were identified, albeit with some differences regarding prevalence of transcripts at each developmental stage. In fact, a low number of copies of *Notch2*, *Dll4*, *Jagged1*, *Jagged2* and *Hes2* transcripts were detected, or were not detected at all, which could be due to individual embryo variability (Menchero et al. 2019) or to the sampling of blastomeres. Although sc-RNAseq is extremely comprehensive in providing data for a single

blastomere, since a whole preimplantation embryo in its later developmental stages can be comprised of many blastomeres (up to 70 in mouse hatched blastocysts), some information may be lost. This proves particularly relevant when analyzing intercellular communication pathways. In this case, the analysis requires both the signal sending and signal receiving cells to be observed, since each signaling status is mutually exclusive (Sprinzak et al. 2010). In the present study, the different early cell lineages – TE and ICM were analyzed as a whole. Although the contribution of each blastomere of a given embryo was not taken into account, the whole picture of signal sending and signal receiving cells in an embryo was able to provide information on whether a particular signaling pathway was active.

Results indicate that, at the individual embryo level, the transcription of Notch components is highly dynamic along development. There is a high variability in the prevalence of some Notch components' transcripts, such as *Notch3*, *Notch4*, *Dll4* and *Hes2*, which indicates that, either these proteins are not critical for physiological developmental processes, or that their translation is being regulated downstream.

The results from transcription analysis studies in early embryos must always be considered with some reserve, since the mammalian embryo initially relies on a large pool of maternally derived mRNA to produce the necessary proteins at the beginning of development. After the maternal-embryo transition (MET), which in the mouse occurs at the 2-cell stage, the embryo begins to transcribe and translate its own genome. However, up to 10% of all RNA present in a blastocyst is maternally derived (Bachvarova and De Leon 1980), having evading translation or active degradation (Barckmann and Simonelig 2013) by a wide range of storage mechanisms (Harvey et al. 2017), such as RNA binding proteins (Kwon et al. 2013) and miRNA (Blakaj and Lin 2008), which were shown to be present at the early embryo stage and necessary for proper embryo development (Bell et al. 2008; Kedde and Agami 2008). This means that the presence of transcripts may not reflect the protein composition of an embryo at a given stage.

To confirm the presence of Notch components in early mouse embryos, an immunocytochemistry approach was used and images were acquired using confocal microscopy to allow a clearer cellular demarcation (Jonkman and Brown 2015), which proves particularly important given the spherical shape of the preimplantation embryo. In the first stage of this work, immunocytochemistry for all four receptors, ligands DLL1, DLL4, JAGGED1 and JAGGED2, and effectors HES1 and HES2 was performed on blastocysts (BL) and receptor NOTCH1 on expanded blastocysts (EBL). The BL developmental stage was chosen for the protein evaluation since it is the first embryo stage when TE and ICM cellular populations are spatially segregated and are more easily identified by their location,

giving a clearer perception if the presence of a given protein is exclusive to either population or is expressed in both. Results showed that, while transcription of *Notch1*, *Notch2*, *Dll1*, *Jagged1* and *Hes1* matched its expression, the presence or absence of transcripts for the other components of this pathway was not consistent with its expression. In fact, BL lacked *Notch4* and *Dll4* transcripts but expressed both these proteins. On the contrary, although 100% of analyzed BL transcribed *Jagged2*, this protein was not found at this stage. Similarly, both NOTCH3 and HES2 were present in BL, although its transcription was only detected in 33% and 40% of embryos of this stage, respectively.

It is possible that NOTCH4 and DLL4 were translated at previous embryonic stages and had not yet been used in canonical signaling, as degradation of receptors and ligands occurs after signal transmission (Wu and Bresnick 2007; D'Souza et al. 2008). Alternatively, even though less likely, NOTCH4 and DLL4 could still be of maternal origin. In fact, DLL4 was detected in mouse oocytes of all follicle developmental stages (Murta et al. 2015) and, since some essential maternally derived proteins can evade active degradation following MET, such as in the case of the subcortical maternal complex (Bebbere et al. 2016), further research is necessary to understand if the DLL4 at this stage is embryo or maternally derived. Conversely, the absence of JAGGED2 in BL was unexpected given the presence of its transcripts in all the analyzed embryos. It is possible that the embryo may be merely storing *Jagged2* and translating it at a later stage, such as the hatched blastocyst (HBL) stage, when *Jagged2* transcription peaks. In this scenario, JAGGED2 may be solely required for embryo-maternal communication and/or *in vivo* hatching, since several Notch receptors and effectors have been identified in the mouse uterine epithelium (Murta et al. 2015).

Our analysis also involved the relative quantification of the transcripts that were ubiquitously present from CM to HBL. Previous studies have shown that if multiple components of a pathway are present and if their transcription/expression is developmentally regulated, there is a high chance that the pathway is active (Wang et al. 2004). In this study we found that *Notch1*, *Notch2*, *Jagged2* and *Hes1* transcription was dynamic throughout development, changing according to embryonic stage, thus predicting a regulatory role for Notch signaling.

The immunostaining here presented confirmed previous reports (Chu et al. 2011; Rayon et al. 2014; Menchero et al. 2019) that detected NOTCH1 in the nucleus of TE cells in BL and EBL. Additionally, nuclear staining was also observed for HES1. Since *Notch1* and *Hes1* transcripts are ubiquitous among embryos, their transcription is dynamic throughout early development, and since both proteins are detected in the nucleus of presumptive TE cells, Notch1 activation may be associated to a constitutive function, as also previously

described (Chu et al. 2011; Rayon et al. 2014), and Notch signaling conveyed by HES1 effector. On the contrary, despite the ubiquitous transcription of *Notch2* at the BL stage, this receptor staining is exclusively cytoplasmic. *Hes2* transcripts were only detected in about half of the analyzed embryos and its staining is also exclusively cytoplasmic. This leads to the conclusion that, at least at the BL stage, this receptor and effector are not responsible for conveying canonical Notch signaling. Additional studies would be valuable to investigate nuclear staining (evidencing activity) of receptors' intracellular domain and Hes effectors at other developmental stages and to explore the presence of other types of Notch effectors, such as the Hey gene family (Fischer and Gessler 2007).

Statistical correlation analysis is used in biological studies to understand if the variation of one variable is related to the variation of another variable (Schober et al. 2018). Although this relationship does not necessarily mean causality, it provides a strong indication that the variables are dependent of each other or at least are being regulated by the same factors. In an attempt to identify a possible canonical Notch signaling pathway, correlation tests were used to investigate the possible relation between the several Notch components. Significant positive correlations were found between transcription of Notch components *Notch1* and *Hes1*, strengthening the hypothesis that canonical signal is conveyed through these proteins.

Notch signaling is known to be involved in pluripotency maintenance or in initiation of the early differentiation processes in many biological scenarios (Bray 2006; Meier-Stiegen et al. 2010; Andersson et al. 2011). Thus, the same correlation analysis was performed to test the hypothesis that Notch signaling could be involved in changes in pluripotency maintenance or initiation of the early differentiation processes observed at this stage, either by directly regulating them or being influenced by them. As follows, Notch components *Notch1*, *Notch2*, *Jagged2* and *Hes1* all correlated with transcription of all analyzed pluripotency and differentiation transcription factors.

Transcription factors SOX2, OCT4 and KLF4 are necessary for the transcriptional activation of the target gene *Nanog* (Wei et al. 2009), and consequently, the maintenance of ICM cellular identity and "stemness" (Huang et al. 2015). Correlations found between Notch and pluripotency transcripts indicate that Notch signaling is potentially responsible for stemness identity and differentiation processes. Therefore, there is a strong indication that mouse early embryonic pluripotency maintenance relies on Notch signaling activation, which was also observed in human embryonic stem cells, where Notch inhibition decreased pluripotent cellular proliferation (Fox et al. 2008) and, more recently, in mouse embryos, where embryonic cells with a loss of function of the Notch pathway either suffered from a

decrease in proliferation ability or increased cell death (Menchero et al. 2019). All these correlations prompt for a regulatory role of the Notch signaling pathway in the embryonic pluripotency maintenance and differentiation processes.

4.2. *In vitro* studies of Notch signaling in preimplantation mouse embryos

Following the confirmation of the presence of Notch components in early mouse embryos and their dynamic transcription indicating activity, a series of *in vitro* assays were designed to understand the exact role of Notch signaling in early embryos. These involved pharmacological activation or inhibition of Notch signaling through treatments that have been extensively used in *in vitro* and *in vivo* biological scenarios (Kared et al. 2006; Li et al. 2008; Jiang et al. 2011; Murta et al. 2014).

Eight to 16-cell embryos were used given that, although the early process of compaction and differentiation has already begun (Marikawa and Alarcon 2012; Schrode et al. 2013), their blastomeres still have enough plasticity to be directed towards the ICM or TE fate (Stephenson et al. 2010; Tarkowski et al. 2010) and are therefore still vulnerable to intercellular signaling disruptions regarding cellular differentiation choices. All treatments were performed until 4.5 dpc, allowing embryo hatching.

4.2.1. Notch signaling activity confirmation – DAPT treatment

To confirm canonical Notch signaling activity in early embryo development, DAPT, a known canonical Notch signaling inhibitor that disrupts γ-secretase activity and indirectly the nuclear translocation of NICD, was used to supplement embryo culture medium. Treatment with DAPT significantly changed embryo developmental kinetics by impairing embryo hatching from 4.0 dpc on. Additionally, transcription analysis revealed that DAPT treatment caused a significant decrease on transcription levels of *Jagged1* on 3.5 dpc BL and of *Hes1* on 4.0 dpc EBL, as seen in other biological scenarios as a consequence of Notch signaling blockade (Ong et al. 2006; Reznikova et al. 2009).

Further effects on embryo transcription were also observed and will be later addressed. Altogether, these results indicate that canonical Notch signaling is active at these embryonic developmental stages, and that it can be disrupted by classical Notch inhibitors.

4.2.2. Notch signaling stimulation – JAGGED1 and JAGGED2 treatment

Transcription analysis results showed a high prevalence and a high relative transcription of *Jagged1* and *Jagged2* in early mouse embryos from the 3.5 dpc CM stage to the 4.5 dpc HBL stage. Also of interest was the evolution of this transcription: while *Jagged1* transcription remained constant and was present in all immunostained BL, *Jagged2* transcription increased to more than double at the HBL stage, although it was still not being translated at the BL stage. Considering the hypothesis that JAGGED1 or JAGGED2 could be involved in Notch signaling activation throughout development, and that these ligands could be involved in pluripotency and differentiation regulation, recombinant proteins JAGGED1 and JAGGED2 were used to supplement *in vitro* culture medium.

Classically, Notch ligands have been used as activators when anchored to a physical medium, such as the extracellular matrix or culture plate, to generate a mechanical force sufficient to pull the receptor-ligand heterodimer apart and activate Notch signaling (D'Souza et al. 2008). However, the endocytosis of Notch ligands that occurs upon receptor-ligand interaction is sufficient to induce Notch activation (Nichols et al. 2007). Nevertheless, to confirm that the obtained results from the ligand recombinant protein supplementation were produced by a true signaling activation and not from a signal blockade derived from receptor saturation with inefficient ligands, the above mentioned DAPT treatment was performed concomitantly. Since embryo kinetics and transcription patterns of ligand treated embryos diverged from both untreated controls and DAPT treated embryos, we can establish that embryo supplementation with the recombinant ligands promoted a true Notch signaling activation and results obtained from this assay could provide information of gain of function.

Supplemented embryos showed similar developmental kinetics to control embryos at 3.5 dpc. However, differences arose in developmental rates between supplemented and control embryos and between JAGGED1 and JAGGED2 supplemented embryos from 4.0 dpc on. Differences at 4.0 dpc were not clear as to whether ligand supplementation increased or decreased embryo developmental progression, as detrimental effects on CM and EBL rates were observed but did not carry on to early hatching rates. Analysis of 4.5 dpc HBL showed a clear detrimental effect of JAGGED2 in the completion of the *in vitro* hatching process, similar to that observed in DAPT treated embryos. In mammalian species, the embryo needs to exit the zona pellucida to attach to the uterus and implant. This process is dependent on mechanical processes, mediated by membranous extensions of the trophectoderm called trophectodermal projections (Gonzales et al. 1996; Seshagiri et al. 2009), contraction and expansion of the blastocyst (Massip et al. 1982), and by molecular regulators among which are transcription factors and cysteine proteases (Seshagiri et al.

2009). The trophectoderm is the key player in these events and an incorrect expression of CDX2, a specific TE transcription marker, leads to a dysfunctional blastocyst that cannot evolve beyond the point of initial cavitation and is unable to expand and hatch (Strumpf et al. 2005; Wu et al. 2010).

Considering the above, an analysis on the transcription pattern of supplemented embryos was performed. Supplementation, either with JAGGED1 or JAGGED2 inhibited the transcription of *Jagged1*, but not of *Jagged2*, uncovering an active negative feedback mechanism by which the embryo controls *Jagged1* levels. Additionally, JAGGED2 supplementation, while not influencing transcription of any other Notch component apart from *Jagged1*, lead to a downregulation of *Cdx2* transcription. This indicates that JAGGED2 supplemented embryos, having no internal or external source of JAGGED1, could not maintain a satisfactory *Cdx2* transcription level, which could explain the observed impairment on blastocyst hatching. Ligand supplementation had no effect on transcription of pluripotency associated factors.

Also noteworthy was the downregulation of *Sox2* transcription in DAPT treated embryos, signifying that NICD cleavage is necessary, not only to maintain normal *Hes1* levels, as expected in canonical signaling, but also influences cellular pluripotency. HES1 is known to participate in the Notch signaling cascade that regulates SOX2 in other biological scenarios (Batchuluun et al. 2017), and results of this work have uncovered a similar mechanism in early mouse embryonic development.

Therefore, a relationship between Notch signaling with both pluripotency maintenance (*Sox2* transcription) through its effector HES1 and with TE differentiation (*Cdx2* transcription) through its ligand JAGGED1 was established. However, since Notch receptors and HES1, which transcription analysis identifies as positive regulators of SOX2, were only detected in the nucleus of presumptive TE cells in BL, and since JAGGED1 transcription does not correlate with other Notch components, it is possible that a non-canonical Notch signaling pathway is being used at this time in early development. Considering the vast amount of signaling pathways active in early embryonic development, and the so far elusive role of some of them, it was hypothesized that Notch could be operating through an interplay with these pathways. In this case, signaling interactions could provide a better understanding of early development than the study of each individual pathway.

4.3. Notch-Wnt interplay in preimplantation development

A good candidate for this interplay with Notch signaling is the Wnt signaling pathway. Wnt activity has been identified in preimplantation mouse embryos and is known to act in a cross-regulatory manner with Notch signaling in many physiological and pathological biological scenarios (Andersen et al. 2012; Collu et al. 2014). This interaction, however, has not yet been shown to occur in early embryo development.

To help clarify the role of canonical Wnt signaling in early mouse embryo development and its putative interplay with Notch signaling, a blockade strategy was used, as in the case of Notch signaling. For this, embryo culture medium was supplemented with the glycoprotein DKK1, which binds to the LRP6 co-receptor with high affinity, preventing the activation of Wnt receptors and the consequent stabilization of β-catenin. Concurring with previous reports (Wang et al. 2004; Xie et al. 2008), results confirmed Wnt activity in early embryos, since DKK1 supplementation decreased β-catenin cytoplasmic pool and its immunostaining was mainly associated with cellular adherens junctions when compared to untreated control embryos. The effectiveness of this strategy to block canonical Wnt signaling was additionally confirmed by the upregulation of transcription of Wnt co-receptor *Lrp6*, in a negative feedback mechanism formerly observed by others (Khan et al. 2007, Li et al. 2010). Most importantly, Wnt blockade produced different embryonic phenotypes of those observed in untreated control embryos regarding embryonic developmental kinetics, differential cell count and gene transcription, as will be discussed bellow.

Although, the use of DAPT and DKK1 produced effective Notch and Wnt signaling blockades, respectively, and the observed phenotypes are indicative of their physiological pathways in embryonic and fetal development, it is necessary to acknowledge that both canonical signaling pathways might have not been fully abolished, but rather significantly decreased. Also, the experimental design, although effective in the study of canonical signaling, does not allow for inferences regarding non-canonical signaling other than Notch-Wnt interplay and, therefore, other non-canonical effects on the observed phenotypes cannot be excluded.

One of the main conclusions of this work is that both Notch and Wnt signaling are responsible for regulating the pace of embryonic development, and that they do so in an opposing manner. Results presented in Chapter II confirm previous observations in Chapter I, where Notch blockade decreased embryonic development, significantly hindering embryo hatching at 4.0 dpc and decreasing, although not significantly, development at 4.5 dpc. From observations in Chapter II it can be concluded that, while Notch stimulates embryo

developmental progress, Wnt halts this progress at 4.0 dpc, at the time of blastocyst expansion.

The regulation of embryonic kinetics is especially important in mammalian species, since embryos need to be precisely coordinated with the maternal window of implantation in order to successfully implant in the uterus and continue their development. This window of implantation (reviewed by Carson et al. 2000) is a very narrow temporal window for embryo-maternal communication (in mice lasts 24 hours; reviewed by Zhang et al. 2013), which onset and end is mainly controlled by the mother (reviewed by Aplin and Kimber 2004). It is only at this time that the uterus will be responsive to embryonic stimulus, and the embryo needs to be at the correct developmental stage to convey such stimulus. As a consequence, while the embryos can wait, although not indefinitely, for the onset of the maternal window of implantation, the maternal uterus will not wait for the embryos to reach the correct developmental stage to implant. It is known that the endometrium secretes DKK1 during the window of implantation in several species, including mice (Kao et al. 2002; Li et al. 2008), and its ability to do so in sufficient amounts has been associated with increased fertility in cows (Minten et al. 2013). Therefore, the uterus itself may be responsible for aiding early embryos pace their development at this critical stage. In this scenario, embryonic Wnt canonical signaling must be downregulated at the stage of blastocyst expansion, allowing faster embryo development towards implantation, while the uterus is still receptive.

Also noteworthy is the fact that it is the balanced action of both Notch and Wnt signaling that is required for a proper physiological development, as the double blockade of Notch and Wnt significantly exacerbated the effects of Notch blockade, slowing development and hindering hatching. This indicates that, while Notch signaling is functioning, Wnt does not need to operate, but in the case of Notch downregulation Wnt may partially compensate its deleterious effects.

Since embryo developmental kinetics is highly dependent on embryo cellular differentiation into ICM and TE cells and their proliferation, the hypothesis that the observed effects of Notch and Wnt blockades could be explained by altering the number of cells of each cellular lineage and / or ICM:TE ratios was investigated. CDX2 immunostaining allowed to differentially count TE cells (blastomeres with positive nuclear staining) and ICM cells (blastomeres with negative nuclear staining). Results evidenced that Notch signaling regulates the ICM:TE ratio in CM and BL, as Notch blockade (alone or simultaneous to Wnt blockade) decreased this ratio, most likely due to a decrease in overall ICM cell count. Notch signaling, together with the Hippo signaling pathway, has been implicated as a positive regulator of CDX2 expression and TE lineage specification in early embryo development

(Rayon et al. 2014; Watanabe et al. 2017; Menchero et al. 2019). The here observed effect on ICM, rather than on TE cells, contrasts with data from Rayon et al. (2014), but is in accordance to what was observed in human embryonic stem cells, where Notch inhibition decreased cellular proliferation, although not affecting its differentiation (Fox et al. 2008). This discrepancy could be explained by the timing of the Notch blockade, since in this study DAPT treatment began at the 16-cell stage during the stochastic patterning of blastomeres, highlighting the previously observed biphasic and dual role for Notch (Menchero et al. 2019) at the very beginning of TE formation and later in the regulation of ICM proliferation and maintenance. Although Wnt has been implicated as a regulator of total embryonic cell number and of TE differentiation in pig (Lim et al. 2013) and bovine blastocysts (Denicol et al. 2013), results from this study did not reveal a significant difference between DKK1 treated embryos and untreated controls in differential cell count. However, a significant difference between DAPT and DKK1 treated BL regarding ICM cell number, which translated in a significantly higher total cell number in DKK1 treated BL, was detected. This observation further stresses the opposing roles of Wnt and Notch signaling at this stage, which culminates in an accelerated development of embryos where Wnt signaling has been blocked and will be discussed bellow.

Signaling pathways operate by regulating key transcription factors that maintain a cellular undifferentiated state or drive them to a given differentiated lineage. The relative amounts of these transcription factors need to be carefully regulated along development and their effect on cellular fate decisions need to be precisely timed, since the negative consequences of their disarrangement carries on to a large number of cells eventually compromising the whole embryo (Sharifi-Zarchi et al. 2015). As such, to understand what transcription factors are being regulated by Notch and Wnt signaling, embryos were analyzed using the whole embryo qRT-PCR. Results confirmed the previous findings that Notch was involved in *Sox2* transcription regulation at the BL stage. Additionally, the blockade of Notch and/ or Wnt signaling produced a deregulation in transcription patterns of other pluripotency associated transcription factors, such as OCT4 and KLF4, and of differentiation associated transcription factor CDX2.

As seen in untreated control embryos, transcription levels of *Sox2* have a physiological rise from the CM to the BL stage as a consequence of the increase in cell number, since all blastomeres at these stages express this protein (Wang et al. 2004; Keramari et al. 2010). Results showed that the blockade of either Notch or Wnt, strongly stimulated the transcription of *Sox2* in CM, increasing it before its physiological rise at the BL stage. Since treatment did not significantly influence CM total cell number, *Sox2* upregulation is due to an increase at the individual blastomere level. Both Notch and Wnt are both equally

responsible for repressing transcription of *Sox2* in CM, until cell fate commitment is fully established beyond this stage. Further confirming a biphasic role for Notch signaling, we observed that Notch is necessary for *Sox2* transcription in BL, since Notch blockade decreased its transcription at this stage, as described in Chapter I and other biological scenarios (Neves et al. 2011; Batchuluun et al. 2017). Additionally, it was evidenced that Notch signaling is a key stimulator of *Klf4* transcription at blastulation, and acts as a transcription repressor by the time blastocysts have matured, as Notch blockade decreased its transcription in BL and increased it in EBL. On the other hand, Wnt blockade alone or simultaneous with Notch blockade altered the pattern of *Oct4* transcription throughout development until the EBL stage. This could also influence ICM pluripotency, since both OCT4 up or downregulation can cause cellular differentiation (Niwa et al. 2000).

Both SOX2 and OCT4 are key regulators of *Nanog* transcription (Rodda et al. 2005), which in turn is essential for maintaining the pluripotent state of ICM cells. KLF4 is another key player in maintaining an adequate self-renewal in ES and ICM cells, by directly interacting with SOX2 and OCT4 (Wei et al. 2009). The disruption of the available amounts of these transcription factors can affect ICM cell pluripotency and function. As such, in this study, Notch signaling alone was essential for a normal embryonic ICM. Also, since we found that double blockade reverses the effect of Notch blockade on blastocysts' *Klf4* transcription, this may indicate that, for this transcription factor, Notch stimulation effect is mediated by Wnt.

Interestingly, the downregulation in *Jagged1* transcription observed at the EBL stage by Notch blockade did not lead to a decrease in *Cdx2* transcription, as seen in JAGGED2 treated embryos. This could be explained by the different developmental stage when *Jagged1* downregulation occurred or by the initial mechanism that promoted this downregulation. It is possible that, although not noticeable by our analysis, JAGGED2 supplementation could have disrupted other elements of the differentiation signaling network or that the negative Jagged1 autoregulation has a different mechanism than that of γ-secretase Notch blockade induced downregulation.

The interaction between both signaling pathways was evident, as their individual blockade altered not only transcription of their own components, but also transcription of the other signaling pathway's components. This is in line with what has been described for Notch and Wnt interaction in other biological scenarios where one pathway induces or inhibits the production of the other pathway components, or where one pathway uses the components of the other (Collu et al. 2014). Results show that Notch blockade downregulated transcription of Wnt co-receptor *Lrp6*, which is the opposite of what is expected when canonical Wnt

signaling is blocked (Li et al. 2010). On the other hand, Wnt blockade downregulated the transcription of Notch effector *Hes1*. Immunocytochemistry results provide an explanation for the observed alteration in transcription, as Wnt (as well as double) blockade increased intra nuclear staining of this effector. It is known that Hes1 has oscillatory transcription and expression patterns and functions in a negative autoregulation loop (Hirata et al. 2002). The complete absence of the diffuse cytoplasmic staining observed in control embryos and intense nuclear staining of DKK1 treated embryos, suggests that in the latter, Hes1 is being the target of an abnormal increase in negative autoregulation.

Another clue to Notch-Wnt interplay is the fact that double blockade induced different effects in embryos than those expected by simple cumulative effects of Notch and Wnt blockades alone. An observed example of this is the transcription of *Jagged1* in BL, where only double blockade was able to downregulate transcription, whereas single Notch and single Wnt blockade had no significant effect compared to control. In this situation, the absence of one signaling was compensated by the other. As such, the observed effects of blockade treatments are the result of Notch and Wnt signaling blockade/activation and their interplay with each other. The same was not observed in the other analyzed developmental stages, highlighting the stage specific function of Notch and Wnt signaling.

From these results it can be concluded that both signaling pathways interplay by inhibiting one another in turns, depending on embryo developmental stage. This biphasic mechanism is observed from the CM stage, where Notch signaling is required, to the EBL stage, where Wnt signaling needs to be repressed. There seems to be two chief elements responsible for Notch interaction with Wnt – Jagged1 and Hes1. Notch ligand Jagged1 is affected by double blockade treatment in a way that is not a cumulative result from single blockades, while *Hes1* transcription is more affected by Wnt and double blockades than Notch blockade alone in EBL. This is in accordance with previous work, which showed that control of *Hes1* promotor can be achieved by the canonical Notch pathway or be mediated by β-catenin (Jin et al. 2009). Also, computational models found that this could induce either a pluripotent “multistable” state or differentiated “monostable” state, respectively (Kay et al. 2017), thus influencing individual and neighbouring cells’ fate decisions.

4.4. Notch-Wnt carry-over effects on embryo-fetal development

To further understand if the deregulation on TE and ICM populations caused by Notch and/or Wnt signaling blockades could have further effects on implantation ability or long term effects in embryo / fetal development, untreated, DAPT and/or DKK1 treated embryos were

transferred to pseudo pregnant females. To investigate embryo implantation ability, females were euthanized at day 5 of gestation (48 hours after embryo transfer) and to assess overall embryo development, gestation was allowed to continue almost to term (which in mice lasts up to 21 days), females being euthanized at day 18 of gestation. It was found that, although Notch and Wnt were only briefly blocked for a period of 36 hours, the alteration on ICM and TE cells' absolute and relative number, as well as the disruptions in pluripotency and differentiation genes' transcription patterns had a significant effect on later fetal development.

At day 5 there were no significant differences in implantation rates among groups, meaning that immediately after treatment BL and EBL were able to maintain proper embryo-maternal communication, attach and implant. As previously said, physiologically, mouse embryos arrive at the uterus at 4.0dpc as CM or early BL (Yoshinaga 2013). The following preimplantation development until the EBL stage occurs in the uterine lumen, and after the escape from the zona pellucida the TE becomes responsible for the direct communication with the uterus (reviewed by Sutherland 2003). The attachment and invasion of the uterine epithelium that occurs in rodents is fast and is complete at 4 days of gestation in mice (reviewed by Wang and Dey 2006). In the embryo transfer assays, only morphologically normal BL and EBL were transferred, regardless of their relative amount in the treatment group. As such, it is unknown if overall implantation rates would be impaired if the developmental rates of the transferred embryos were representative of their original treatment group. It is likely that the less developed embryos, namely from the Notch and double blockades, would not reach implantation competence in time.

In contrast, signaling blockades had a significant effect on embryo development to term. While Wnt blockade had no effect on the rate of fully developed fetuses, Notch blockade significantly decreased fetal development until term. Further highlighting the interplay between Notch and Wnt signaling pathways, double blockade aggravated the effects of the loss of Notch signaling, meaning that Wnt signaling could not operate to partially rescue fetal development. It is therefore clear that Notch and Wnt interplay has a major role in regulating early embryonic development and that flaws in their early actions have permanent consequences on fetal development.

Also of consequence is the observation that Notch and Wnt blockades at the early stages of first cell differentiation had a significant effect on fetal weight. A decrease in final fetal weight can be explained either by a low number or function of ICM cells (Binder et al. 2012; Noli et al. 2015), or indirectly by a dysfunctional placenta, translating TE derived trophoblast cellls' function (Jansson and Powell, 2007). Additionally, it is important to note

that trophoblast proliferation at the time of implantation is also dependent on ICM function (Ansell and Snow 1975).

The reduced ICM cell number and the reduced ICM:TE ratio observed in the Notch blockade group can explain the reduced fetal growth, as the handicapped ICM of these embryos produced smaller fetuses, as previously observed (Binder et al. 2012). On the other hand, and again displaying the opposing roles of Notch and Wnt signaling, Wnt blockade produced significantly heavier fetuses than the control group. Denicol et al. (2014) observed a similar effect in DKK1 treated bovine embryos, reporting that treatment tended to produce larger fetuses at 34 days of gestation. Confirming above effects, embryos from simultaneous Notch and Wnt blockades, showed no significant differences in weight from those of the control group. Therefore, it can be concluded that there was a beneficial effect from silencing Wnt signaling at that point, although the results were only seen at a much later stage, possibly from healthier ICM cells. However, and since the fetuses were not allowed to be born, it is not possible to infer about the resulting newborn's viability, nor can be excluded the possibility that the weight increase observed is similar to the ART-derived large offspring syndrome observed in other mammalian species with mainly singleton pregnancies (Tunster et al. 2011).

4.5. Conclusion

The present work characterized the Notch signaling pathway in early mouse embryos and uncovered features of its role in the first cell differentiation events, which have carry-over effects on the development of a fully formed fetus. It was evidenced that Notch signaling components, such as receptors Notch1 and Notch2, ligands Jagged1 and Jagged2 and effector Hes1, are present during the early stages of development, from the 3.5 dpc CM to the 4.5 dpc HBL stage, and their transcription and expression patterns are dynamic along development. Their correlations with pluripotency and differentiation associated transcription factors, together with the active status of the pathway, indicates that Notch signaling is an important hub in the regulation of early embryo development.

Through Notch activation experiments using recombinant Jagged ligands, it was uncovered a relevant role for Jagged1 in regulating *Cdx2* transcription at the BL stage and embryo hatching competence. Moreover, using a canonical Notch signaling blockade strategy, a role for Notch in maintaining ICM pluripotency status and proliferation ability, which is necessary for proper fetal development to term, was uncovered. As observed in other biological scenarios, it was also uncovered an active interplay between Notch and Wnt

signaling in early embryos, which maintains a balanced embryo developmental kinetics, as well as hatching and implantation ability. This work evidenced that Wnt signaling blockade has no major detrimental effects on mouse early embryo development, and appears to be beneficial for embryo kinetics and future fetal development. However, in the case of Notch signaling abrogation, Wnt signaling can reduce the deleterious effects of Notch absence, as double blockade leads to a significant loss in embryo ability to implant and develop to term, highlighting the necessity for both in the complex environment of the embryonic regulatory signaling network.

This work is germinal for future studies involving the advancement of strategies to increase both human and animal embryo production success rates and eventually rescue suboptimal *in vitro* produced embryos, by manipulating Notch and Wnt signaling pathways. Further research is necessary to fully uncover the molecular mechanisms behind early embryonic Notch and Wnt signaling interplay and to precisely characterize the consequences of its manipulation in other animal and human species.

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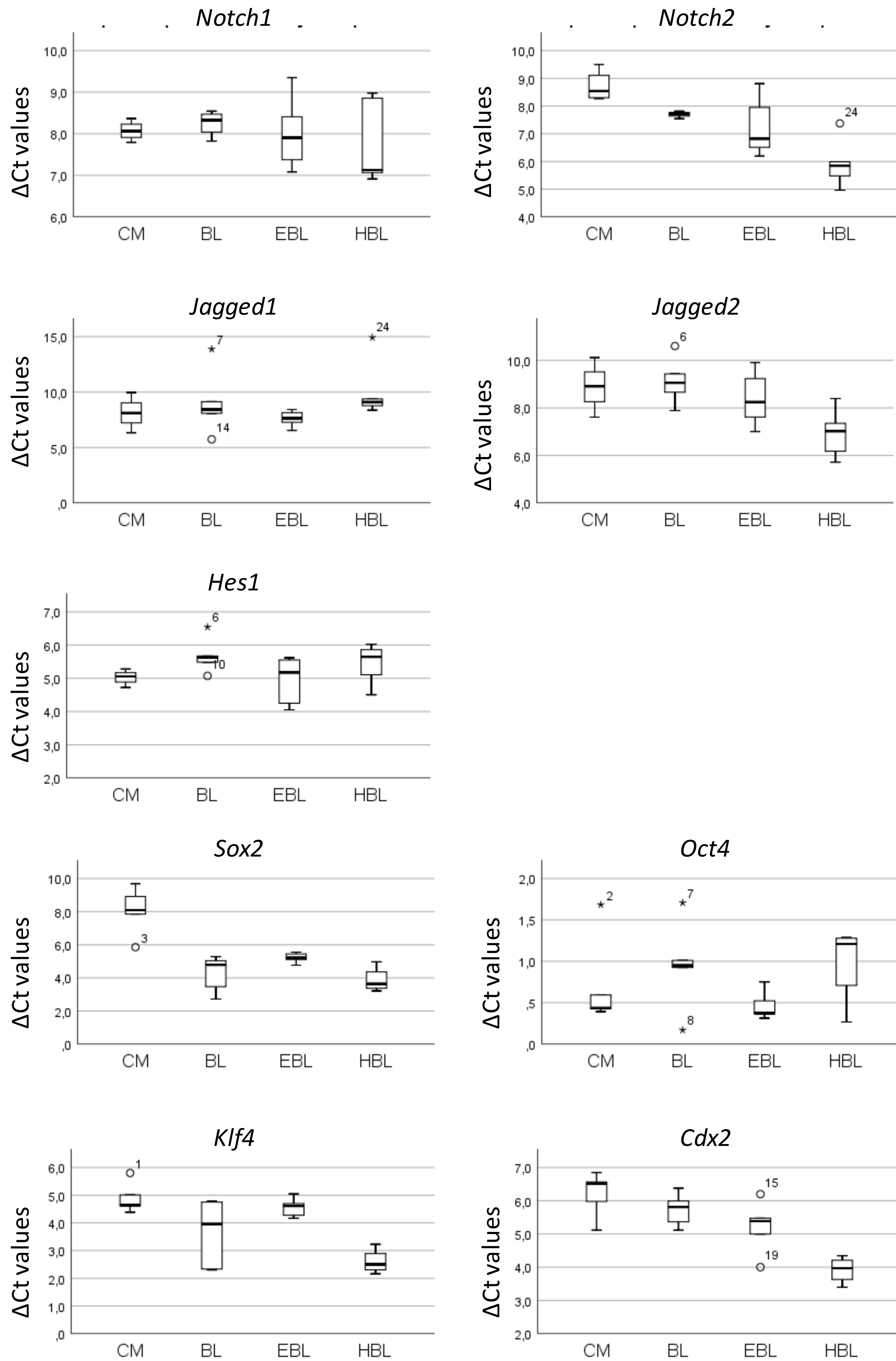
6. ANNEX I: Primer pair sequences list

Target gene	Sequence (5' – 3')	Product length (bp)	Accession no.
Notch1	Fwd: ACAGTAACCCCTGCATCCAC Rev: GGTTGGACTCACACTCGTTG	120	NM_008714.3
Notch2	Fwd: GACTGCACAGAAGACGTGGA Rev: GCGTAGCCCTTCAGACACTC	116	NM_010928.2
Notch3	Fwd: GTGTCAATGGTGGTGTCTGC Rev: GCACACTCATCCACATCCAG	103	NM_008716.2
Notch4	Fwd: GAGGGACACTCCACCTTTCA Rev: CTGGTGCCTGACACAGTCAT	93	NM_010929.2
Delta-like1	Fwd: GTTGTCTCCATGGCACCTG Rev: TGCACGGCTTATGGTGAGTA	111	NM_007865.3
Delta-like4	Fwd: GGAACCTTCTCACTCAACATCC Rev: CTCGTCTGTTCGCCAAATCT	141	NM_019454.3
Jagged1	Fwd: CCAGCCAGTGAAGACCAAGT Rev: CAATTCGCTGCAAATGTGTT	127	NM_013822.5
Jagged2	Fwd: AGTGCCATCTGGCTTTGAAT Rev: CGCTGCACATGGGTTAGAG	97	NM_010588.2
Hes1	Fwd: GCGAAGGGCAAGAATAAATG Rev: TGTCTGCCTTCTCTAGCTTGG	104	NM_008235.2
Hes2	Fwd: CGGATCAACGAGAGCCTAAG Rev: GTCTGCCTTCTCCAACTTCG	93	NM_008236.4
Sox2	Fwd: GGTTCTTGCTGGGTTTTGATTCT Rev: CCTTCCTTGTTTGTAACGGTCCT	59	NM_011443.4
Klf4	Fwd: GCAGTCACAAGTCCCCTCTC Rev: GACCTTCTTCCCCTCTTTGG	93	NM_010637.3
Oct4	Fwd: TGGAGGAAGCCGACAACAAT Rev: GCTGATTGGCGATGTGAGTG	177	NM_013633.3
Cdx2	Fwd: CTGGCTCCGCAGAACTTTGT Rev: GGTGCGTAGCCATTCCAGTC	170	NM_007673.3
Wnt3a	Fwd: CATCTTTGGCCCTGTTCTGG Rev: GCGTGTCACTGCGAAAGCTA	91	NM_009522.2
Lrp6	Fwd: CAAGCTGCTGGAGAATGGAAA Rev: ATGGCATGCCGGATATCTTCT	147	NM_008514.4
Cdca7	Fwd: ACA TGC TGG TGA GAC AGA GGA A Rev: TAT ATG CGG AAG GGT CAT GGA	98	NM_025866.3
Lgr5	Fwd: CCC ATC CAA TTT GTT GGA GTA Rev: GTG GCA GTT CCT GTC AAG TG	113	NM_010195.2
Rps29	Fwd: CACGGTCTGATCCGCAAATAC Rev: ACTAGCATGATCGGTTCCACTTG	144	NM_009093.2
Hprt1	Fwd: GTCGTGATTAGCGATGATGAACC Rev: GCAAGTCTTTCAGTCCTGTCCATAA	128	NM_013556.2

7. ANNEX II: Boxplot of ∆Ct values obtained from gene prevalence analysis



Boxplot of ∆Ct values of transcription levels of Notch and pluripotency and differentiation genes in 3.5 dpc compact morulae (n=9), blastocysts (n=9) and expanded blastocysts (n=7), and in 4.5 dpc hatched blastocysts (n=5). Ct values of target genes were normalized to the average of Ct of housekeeping genes *Rps29* and *Hprt1*.