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A regulatory crosstalk in Down syndrome: competing mRNA-miRNA network

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1 Abstract (Italiano)

La sindrome di Down (DS), la cui causa genetica fu ricondotta alla trisomia completa o parziale del cromosoma 21 (HSA21), è la più comune aneuploidia umana compatibile con la vita. La DS è una complessa condizione genetica caratterizzata da più di 80 diversi fenotipi clinici, con espressività e penetranza estremamente variabili. Gli individui affetti da DS presentano alterazioni - sia di carattere strutturale sia funzionale - a carico di differenti organi e sistemi, che lasciano presupporre un’alterazione dell’embriogenesi negli individui affetti da sindrome di Down, dovuta alla Trisomia 21. L’alterazione del quadro fenotipico in individui con la sindrome di Down è stata attribuita in primo luogo ad anomalie nel dosaggio genico, derivanti dalla trisomia del cromosoma 21. Tuttavia, studi di espressione genica eseguiti su larga scala hanno messo in luce uno scenario più complesso, in cui si assiste a una deregolazione trascrizionale globale, estesa ai geni euploidi. In tale contesto, è ipotizzabile l’esistenza di un meccanismo di controllo dell’espressione genica più complesso, che presuppone interazioni dirette e/o indirette tra i prodotti genici del cromosoma 21 e i geni localizzati sugli altri cromosomi. Inoltre, recentemente è emerso un cross-talk tra RNA messaggeri, pseudogeni e lincRNA, mediato dalla competizione per il legame a specifici miRNA. Pertanto, lo sbilanciamento genico dovuto alla trisomia del cromosoma 21, potrebbe agire sull’espressione genica globale, mediante un cross-talk mediato dall’interazione con specifici miRNA durante l’embriogenesi, causando alterazioni dello sviluppo e determinando, conseguentemente, il carattere multisistemico tipico del fenotipo della sindrome di Down.

Alla luce di tali evidenze, lo scopo di questo progetto di dottorato si è focalizzato sullo studio della deregolazione genica globale e del potenziale ruolo di “spugne” di microRNA svolto dai geni HSA21 sovraespressi nella sindrome durante lo sviluppo embrionale.

L’analisi di dataset pubblici di espressione di miRNA e mRNA condotti su embrioni umani alla 4-6 settimana di sviluppo ha permesso di delineare un
network di interazione miRNA/mRNA durante l’embriogenesi. Inoltre, tale studio ha mostrato che la sovraespressione del 3’UTR del gene HSA21 HUNK, sovraespresso nella sindrome di Down, induce un aumento dell’espressione di geni coinvolti nello sviluppo embrionale, tra cui BCL2, CLIC5, EPHA5, ERBB4, HIPK2, MECP2, ONECUT2, e WNT5A, ed una riduzione dell’espressione di miRNA, tra cui miR-17, miR-20a, miR-20b, miR-128 e miR-200c. Tali risultati suggeriscono che ci siano perturbazioni del normale network di regolazione tra mRNA e miRNA durante lo sviluppo di embrioni affetti da DS, causate dalla sovraespressione dei geni HSA21 presenti in triplice copia. Ciò spiegherebbe, almeno in parte, le alterazioni a carattere multisistemico tipiche della sindrome.
2 Abstract (English)

Down syndrome (DS) - also known as Trisomy 21 - is a genetic disorder caused by an extra copy of all or part of human chromosome 21 (HSA21). DS is a complex genetic condition characterized by over 80 clinically different phenotypes of variable penetrance and expressivity. Individuals with DS show alterations - both structural and functional - affecting distinct organs and systems, which suggest that a perturbation of embryogenesis occurs in individuals with Down syndrome, due to Trisomy 21.

Large-scale gene expression studies have revealed a more complex scenario, highlighting a global transcriptional deregulation, extended also to genes mapping outside the DS Critical Region (DSCR) on HSA21, as well as on the other chromosomes. In this context, it is clear that direct and/or indirect interactions between gene products of HSA21 and those from the other chromosomes can better explain the complexity of the clinical manifestations of the disorder. Recent studies have pointed out a previously unexpected role of non-coding RNA (ncRNAs), such as pseudogenes and lincRNAs, on gene expression regulation. In particular, a new crosstalk mediated by the competition for the binding to specific miRNAs has been demonstrated between messenger RNAs and ncRNAs. Such a regulatory crosstalk represents a new and interesting "RNA language" through which different mRNAs regulate each other by competing for miRNAs' availability.

Given these recent findings and considering that Trisomy 21-induced gene imbalance perturbs the entire transcriptome and occurs throughout the embryogenesis, the above-described regulatory crosstalk may be altered by HSA21 trisomy in DS foetuses. The pathological overexpression of HSA21 genes may perturb gene expression during embryogenesis through the alteration of the crosstalk mediated by the interaction with specific miRNAs. In turn, it would cause typical multisystem clinical manifestations of Down syndrome.
Thus, the aim of this PhD project is to deeply explore the global gene deregulation, and the potential role of microRNAs' "sponges" played by HSA21 genes that are overexpressed in DS during embryonic development. Taking advantage of publicly available mRNA and miRNA expression datasets of human embryos (4-6 weeks), a regulatory miRNA/mRNA network in these crucial weeks of the human embryonic development was established. A significant fraction of genes within the network belong to developmental-related pathways. Afterwards, HSA21 genes overexpressed in DS embryos that belong to this network were identified using RNA-Seq datasets from DS and euploid matched chorionic villi. HUNK gene was computationally selected as the best candidate to be an HSA21-derived miRNAs’ sponge. Experimental studies confirmed that the overexpression of its 3'UTR induces an increased expression of genes involved in embryonic development, including BCL2, CLIC5, EPHA5, ERBB4, HIPK2, MECP2, ONECUT2, and WNT5A, and a reduction of the expression of correlated miRNAs, including miR-17, miR-20a, miR-20b, miR-128 and miR-200c. Although further studies are still in progress, the results of both the computational and the experimental studies strongly suggest that the overexpression of HSA21 genes may perturb the physiologic regulatory miRNA/mRNA network during DS embryos' development. This would explain, at least in part, the multisystemic nature of the alterations that typically occur in individuals with the syndrome.
Introduction

3 Introduction

3.1 Down syndrome

Down syndrome (DS) - also known as Trisomy 21 - is a genetic disorder caused by an extra copy of all or part of human chromosome 21 (HSA21; Figure 1). DS was first characterized as a separate form of mental disability in 1866 by John Langdon Down, who used the term ‘mongoloid’, due to shared facial similarities of children with Down syndrome with those of Mongolian race (Down, 1995). The term ‘Trisomy 21’ began to be addressed to DS in 1959, when Jérôme Lejeune reported the discovery that Down syndrome resulted from an extra chromosome, using karyotype technique (Lejeune et al., 1959). Trisomy 21 is the most frequent human aneuploidy with an incidence of 1 in every 700-800 live births in Western countries (Park and Chung, 2013).

Figure 1: Trisomy 21 karyotype showing three copies of human chromosome 21 (HSA21)
3.1.1 Down syndrome as a multisystem disorder

DS is a complex genetic condition characterized by over 80 clinically different phenotypes of variable penetrance and expressivity. Individuals with DS show alterations - both structural and functional - affecting distinct organs and systems, which generally evolve with age (Antonarakis and Epstein, 2006). Physical growth delays, distinguishing dysmorphic features - especially visible in the craniofacies, hands and feet - muscle hypotonia, and mild to moderate intellectual disability represent constant features observed in DS. Individuals with DS present an increased risk of early-onset Alzheimer-like neurodegeneration (Reeves et al., 2001; Wiseman et al., 2009). Two groups of major congenital abnormalities, that can cause morbidity or even death if left untreated, are associated with DS: congenital heart disease (CHD) and several types of gastrointestinal-tract obstruction or dysfunction. CHD affect approximately 50% of children with DS, with a wide range of defects and severity. Atrial or ventricular septal defect, complete atroventricular-canal defect, mitral valve problems, tetralogy of Fallot and patent ductus arteriosus are common features of DS heart (Antonarakis and Epstein, 2006; Wiseman et al., 2009). The gastrointestinal system is also affected, particularly duodenal stenosis or atresia, pyloric stenosis, Meckel diverticulum, imperforate anus and Hirschprung disease occur with an increased incidence in DS children (Patterson, 2007). Moreover, defects involving the immune and haematopoietic systems are recurrent phenomena in the pathogenesis of the syndrome. Individuals with Down syndrome have a 20-fold increased risk of childhood acute lymphoblastic leukaemia (ALL) and a decreased risk of solid tumours, such as breast cancer, in all age-groups (Patterson, 2009). These evidences, combined to the reduced incidence of diabetic proliferative retinopathy and atherosclerosis, strongly indicate a suppression of angiogenesis (Ryeom and Folkman, 2009).
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3.1.2 Embryogenesis and Down syndrome

The multisystemic nature of DS phenotype suggests that Trisomy 21 has a significant impact on the development and differentiation of several organs and tissues. It is reasonable that the presence of three copies of HSA21 genes in the early stages of DS embryonic development determine alterations of organogenesis. Experimental approaches to elucidate the mechanisms by which aneuploidy affects development represent a considerable challenge. Since systematic studies of embryonic and fetal development are difficult in human, mouse models and embryonic stem (ES) cells have been used to study the role of mammalian aneuploidy in developmental processes. Many genetic pathways regulating development are affected in similar ways in DS and DS mouse model (Baxter et al., 2000). Several evidences have revealed that Trisomy 21 affects development of brain, neuronal synapses, cerebellum, neurocranium, heart, mandible, and eye. Indeed, delays in neocortical development and delays in prenatal growth of cerebral cortex and hippocampus, due to reduced neurogenesis from the ventricular zone neural precursor population, were found in DS mouse models (Chakrabarti et al., 2007). Moreover, cerebellar volume is significantly reduced in Ts65Dn mice - due to reduction of both the internal granule layer and the molecular layer of the cerebellum - and craniofacial abnormalities are seen in DS and in the Ts65Dn and Ts1Cje mouse models (Baxter et al., 2000; Richtsmeier et al., 2002). Regarding the heart development, abnormal ventricular septation, representing a failure of fusion between the ventricular septum and the proximal outflow tract cushions, was seen in Tc1 mice (O’Doherty et al., 2005).

3.1.3 Chromosome 21 and gene expression

DS pathological features are considered to be a direct consequence of the dosage imbalance, and in turn of gene expression levels alterations, of genes located on chromosome 21. However, several genetic factors, such as different allele combinations of HSA21 genes, and environmental influences are likely to contribute to individual variability in DS.
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The phenotypic alterations occurring in individuals affected with DS was primarily attributed to gene dosage imbalance resulting from the presence of an extra copy of chromosome 21. Thus, characterization of all HSA21 genes represented a starting point to better understand the molecular mechanisms underlying the different phenotypic manifestations of the DS.

Human chromosome 21 is the smallest acrocentric chromosome and represents 1.5% of the entire human genome, spanning about 50 million base pairs. The almost complete, high-quality sequence of the long arm (21q) of HSA21 was published in 2000 (Hattori et al., 2000). The initial analysis of 21q revealed 225 genes (127 known genes and 98 putative novel genes predicted in silico) and 59 pseudogenes (Hattori et al., 2000). Although the precise gene catalogue has not yet been conclusively determined, the total number of HSA21 genes has increased to more than 350 (Gardiner, 2003). Several studies have been focused to find out specific genotype-phenotype correlations, through the characterization of chromosome 21 genes functions and the consequences of their increased expression to specific features of the phenotype, according to a 'gene dosage' hypothesis. Such hypothesis states that specific phenotypes are a consequence of a gene dosage imbalance that results in overexpression of individual causative genes. To address this issue, human partial trisomy and DS mouse models have been studied (Lyle et al., 2009). Mapping partial HSA21 trisomy allowed narrowing studies to a limited region of chromosome 21, called “Down Syndrome Critical Region” (DSCR), that contains genes responsible for many features of DS (Barlow et al., 2001; Korenberg et al., 1990; Sinet et al., 1994). The DSCR was defined with a proximal boundary between markers D21S17 (35 892 kb) and D21S55 (38 012 kb), and a distal boundary at MX1 (41 720 kb). This is a region spanning 3.8–6.5 Mb and containing 25–50 genes. Although the notion of a DSCR has gained some acceptance in DS research, the data supporting it remain controversial. The generation of several DS mouse models enabled further progress in the research for dosage-sensitive genes underlying DS. HSA21 shares conserved
synteny with orthologous regions on three mouse chromosomes: Mmu10, Mmu16 and Mmu17 (Figure 2).

Figure 2: HSA21 conserved synteny with mouse chromosomes Mmu10, Mmu16 and Mmu17

The first two models that were generated, Ts65Dn and Ts1Cje mice, with duplications of parts of Mmu16, showed several morphological and functional features of DS. In particular, it was demonstrated that some DSCR genes are involved in development of different organs and tissues. Indeed, the presence of three copies of the *DYRK1A* gene is related to learning, memory and brain development, neurodegeneration, and motor control, while *DSCAM* gene is associated to cardiac defects and *ETS2* and *ERG* genes are involved in leukaemia (Lana-elola et al., 2011).

However, the strategy to map partial trisomy 21 in animal models or to analyze by microarray the gene expression in different DS cells/tissues, have often reported conflicting results (Diller et al., 2008; Jablonska et al., 2006; Li et al., 2006; Lyle et al., 2009; Sommer and Henrique-Silva, 2008). Indeed, a study on a mouse model either trisomic or monosomic for the syntenic DSCR found that trisomy for the DSCR alone is not sufficient to produce the structural and
Introduction

functional brain phenotypes (Olson et al., 2007). These accumulating evidences highlight a more complex scenario, suggesting fundamental pieces of the Down syndrome genotype-phenotype jigsaw puzzle are still missing.

3.2 Beyond the gene dosage hypothesis

The simplest model for levels of gene expression in DS would predict that each gene on chromosome 21 would be expressed at 150% with respect to expression levels in euploid individuals. Thus, gene-expression differences between euploid and trisomy 21 cells and tissues have been studied by microarrays, serial analysis of gene expression (SAGE) methodology, differential display PCR, or Real-Time quantitative PCR (FitzPatrick et al., 2002; Kahlem et al., 2004; Lyle et al., 2004; Mao et al., 2003; Saran et al., 2003). Such studies have often reported conflicting results. Indeed, several studies showed that only 37% of genes in various DS tissues were expressed at the theoretical level of 1.5-fold relative to euploid. Therefore, HSA21 genes could show an expression level different from the expected 1.5 fold, implying the existence of a compensation mechanism of gene dosage (Kahlem et al., 2004; Lyle et al., 2004).

Moreover, large-scale gene expression studies performed on DS specimens have revealed a more complex scenario, highlighting a global transcriptional deregulation, extended to euploid genes (Costa et al., 2010, 2011; Esposito et al., 2008; FitzPatrick et al., 2002; Vilardell et al., 2011). Hence, although strong evidences suggest that the dosage imbalance of specific HSA21 genes directly contributes to some characteristic features of the Down syndrome, additional mechanisms are needed to fully explain the complexity of the manifestations and the global gene deregulation of the disorder. Such global mechanisms may include copy number variations, transcription factors alteration, conserved non-coding regions, post-transcriptional regulation, DNA methylation and gene-gene interactions (Patterson, 2007). For example, at least 25 genes localized on chromosome 21 encodes proteins that directly or indirectly regulate gene
transcription, while other chromosome 21 proteins contribute to post-translational modification of transcription factors, including their phosphorylation, dephosphorylation and sumoylation (Gardiner, 2006). Thus, altered stoichiometry in transcription factors’ complexes, due to extra copy of HSA21, may contribute to the Down syndrome phenotype, determining perturbation of downstream gene expression. Moreover, recent studies have highlighted the role of non-coding RNAs (ncRNAs) in regulation of gene expression. Particularly, microRNAs (miRNAs) have been shown to control gene expression, interfering with translation or inducing degradation of mRNA. Further, miRNAs have been identified in all tissues in mammals and have been associated with several biological processes, including development and differentiation (Motti et al., 2012). Since each miRNA may regulate expression of hundreds mRNAs and five miRNAs (i.e., miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) are encoded by genes on HSA21, it is reasonable that HSA21-derived miRNAs could control expression of other genes. Indeed, HSA21-derived micro-RNAs are overexpressed in Down syndrome brain and heart specimens, leading to improper repression of specific target proteins that are linked to specific phenotypes (Dierssen, 2012).

In conclusion, overexpression of individual genes cannot be considered independently when one considers phenotype–genotype correlations. Indeed, global gene deregulation suggests a more complex mechanism of gene expression regulation, which involves direct and/or indirect interactions among HSA21 gene products and genes located on the other chromosomes (Figure 3).
3.2.1 ceRNA hypothesis

Recently, several evidences have shown cross-talk between RNAs, both coding and non-coding, through microRNA binding, which determine large-scale regulatory network across the transcriptome (Poliseno et al., 2010; Salmena et al., 2011; Tay et al., 2011). RNAs influence each other’s levels by competing for a limited pool of miRNAs. This competing endogenous RNAs (ceRNAs) act as miRNAs “sponges” by sharing common microRNA responsive elements (MREs), inhibiting normal miRNAs activity. These sponges absorb miRNAs, lowering the levels of available miRNAs for the target mRNA, resulting in increased translations (Figure 4).
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This new “RNA language" mediated by MREs regulates gene expression in several biological processes, including development and differentiation. Thus, ceRNA activity explains the complexity of organisms' transcriptome landscape. Moreover, perturbations of ceRNAs and ceRNA networks could have an impact on disease onset. Indeed, ceRNAs have been shown to be involved in the pathogenesis of several common cancers such as melanoma, prostate cancer, liver cancer, breast cancer, and thyroid cancer (Fan et al., 2013; Kumar et al., 2014; Tay et al., 2011, 2014).

3.3 microRNAs

In the past ten years, genome-wide expression analyses have revealed a pervasive transcription of human genome, bringing to light thousands of non-coding transcripts. These evidences determined a revolution in the “RNA world", leading to reconsider the central dogma of biology (Mattick and Makunin, 2006). Further studies have revealed that ncRNAs represents an
hidden layer of molecular genetics, which fulfill a wide range of functions, including the control of chromosome dynamics, splicing, RNA editing, translational inhibition and mRNA destruction (Eddy, 2001).

A class of ncRNAs is represented by microRNAs, which are short (22 nucleotide in length) endogenous non-coding RNAs involved in gene expression regulation in plant and animals. The first miRNA, *lin-4*, was discovered in 1993 in *C. elegans*. *lin-4* gene did not encode a protein, but it produced a short non-coding RNA complementary to multiple sequences in the 3’ UTR of the *lin-14* gene, which control the timing of larval development by repressing *lin-14* (Lee et al., 1993). Nevertheless, only a few years later, the characterization of a second small ncRNA, *let-7*, highlighted the hidden role of miRNAs regulation (Reinhart et al., 2000).

miRNAs influence gene expression by sequence-specific binding to target mRNAs and promoting their degradation and/or translational inhibition (Bartel, 2004). miRNAs have been shown to play a crucial regulatory role in several biological processes, including developmental transitions, neuronal patterning, apoptosis, adipogenesis metabolism and hematopoiesis. Moreover, altered levels of miRNAs’ expression have been associated with the pathogenesis of different human diseases, such as cancer, cardiovascular disorders, Alzheimer disease, viral infections and metabolic diseases (Singh et al., 2008).

### 3.3.1 miRNA biogenesis

Most miRNA genes are transcribed by RNA polymerase II into a long primary transcript (pri-miRNA) with a local hairpin structure containing miRNA precursor, pre-miRNA (Ha and Kim, 2014; Figure 5).
Pri-miRNA can range in size from hundreds of nucleotides to tens of kilobases (Bushati and Cohen, 2007). However, miRNAs can also be transcribed by RNA polymerase III, which specifically synthesizes small non-protein coding RNAs, such as tRNAs, 5S ribosomal RNA, and the U6 snRNA (Bartel, 2004). Most mammalian miRNAs are intergenic or are encoded by introns of both coding and non-coding genes, while some miRNAs are encoded by exonic regions. Intrinsic miRNAs’ processing does not affect splicing of the host pre-mRNA and appears to occur co-transcriptionally before splicing catalysis (Kim et al., 2007).

The pre-miRNA hairpin is processed from the pri-miRNA transcript within the nucleus by a multiprotein complex called the Microprocessor. The core of this complex consists of the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein known as DiGeorge syndrome critical region 8 (DGCR8 or "Pasha" in invertebrates) (Ha and Kim, 2014). DGCR8 recognizes the pri-miRNA at the junction of single stranded RNA and double
stranded RNA and guides the catalytic RNase III domain of Drosha to a specific cleavage. Drosha cleaves the pri-miRNA ~11 bp from the junction on the stem, producing a ~ 60-70 bp pre-miRNA with a 5’ phosphate and a two-nucleotide 3’ overhang (Han et al., 2006). Following Drosha processing, pre-miRNA is exported into the cytoplasm to complete maturation process. The export receptor Exportin-5 recognizes the 2-nt 3’ overhang, characteristic of RNase III-mediated cleavage, and transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism (Ha and Kim, 2014). Once in the cytoplasm, the pre-miRNA is cleaved near the terminal loop by Dicer to produce the mature ~22-nt miRNA:miRNA* duplex (Bushati and Cohen, 2007). Dicer is another RNase III endonuclease, highly conserved in all eukaryotes, with a N-terminal ATPase/Helicase domain, DUF283 (domain of unknown function), PAZ (Piiwi/Argonaute/Zwilli) domain, and two tandem RNase III nuclease domains (RNase IIIa and RNase IIIb) located at the C-terminal followed by a dsRNA-binding domain (dsRBD). The PAZ domain binds the 2nt 3’ overhang of pre-miRNA and the two RNase III domains cleave (“dice”) the dsRNA (Macfarlane and Murphy, 2010).

Dicer, together with the human immunodeficiency virus trans-activating response RNA binding protein (TRBP), recruits the Argonaute protein Ago2 (and other Ago proteins), and they form a trimeric complex that initiates the assembly of the RNA-induced silencing complex (RISC), a ribonucleoprotein complex (Bushati and Cohen, 2007). Only one strand is usually incorporated into RISC complex: the miRNA strand with relatively lower stability of base-pairing at its 5’ end is incorporated into RISC, whereas the miRNA* strand is typically degraded. Once incorporated into RISC, the miRNA guides the complex to its mRNA targets for silencing mediated by base-pairing interactions.

### 3.3.2 miRNA-mediated silencing

miRNAs mediate mRNA silencing through physical interaction with 3’UTR sequence of mRNA. Target silencing may occur either via mRNA degradation
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or preventing mRNA translation, depending on the identity of base pairing. In cases of perfect complementarity to the miRNA, target mRNAs undergo degradation; otherwise, their translation is prevented (Bushati and Cohen, 2007). miRNAs mediate mRNA repression by recruiting the RISC complex to target mRNAs. Argonaute proteins, specifically Ago2 in mammals and flies, are capable of endonucleolytic cleavage and are essential for miRNA-mediated gene silencing (Bushati and Cohen, 2007; Fabian and Sonenberg, 2012). Ago proteins interact with GW182/TNRC6 (TriNucleotide repeat-containing) family proteins, which provide a platform for interaction with several proteins and complexes required for miRNA target decay (Fabian and Sonenberg, 2012). First, interaction with poly(A) binding protein (PABP), and CCR4-NOT and PAN2-PAN3 deadenylase complexes, induces target deadenylation of the poly(A) tail. Following deadenylation, DCP1-DCP2 decapping complexes remove the 5’ terminal cap and the target mRNA is degraded by Xrn1 5’-3’ exonuclease (Fabian and Sonenberg, 2012; Inada and Makino, 2014). Imperfect base-pairing of miRNAs with their targets promotes translational repression rather than cleavage and degradation. The mechanism of translational repression by miRNAs remains controversial. Some studies show that miRNAs block translation initiation, whereas other studies suggest a block in elongation (Bushati and Cohen, 2007). A recent model suggests that during miRNA-mediated repression all three levels of translation (initiation, elongation and termination) are coordinately inhibited or slowed (Gu and Kay, 2010; Figure 6).
Indeed, RISC complex inhibits translation initiation by interfering with eIF4G cap recognition, by preventing the circularization required for efficient translation and by preventing the 60S and 40S ribosomes assembly. Moreover, miRNA-induced silencing complex might inhibit ribosome elongation or promote premature translation termination and co-translation degradation in the P-bodies (Bushati and Cohen, 2007; Gu and Kay, 2010).

Figure 6: Mechanisms of miRNA-mediated silencing

Sethi et al., 2013
4 Aim

The multisystemic nature of Down syndrome suggests the involvement of chromosome 21 trisomy in the development and differentiation of different organs and tissues (Wiseman et al., 2009). However, the role of triplicated genes and the mechanism by which they alter the events underlying organogenesis are still unclear. Thus, addressing the pathogenic mechanisms underlying the Down syndrome in the early stages of embryonic development, when organogenesis occurs, is of crucial relevance. Moreover, large-scale gene expression studies on Down syndrome cells/tissue – carried out also in our laboratory (Costa et al., 2010, 2011) - revealed a more complex scenario. Particularly, it has been shown that the transcriptional deregulation is not limited to genes mapping on HSA21, but it is extended to euploid genes, suggesting direct and/or indirect interactions between HSA21 genes and genes located on other chromosomes. Furthermore, very recently, the existence - at least in tumours - of a new gene regulatory mechanism based on the competition for microRNAs binding (the competing endogenous RNAs, ceRNAs) has been demonstrated.

In light of these considerations, the aim of my PhD project is to understand whether the multisystemic alterations of Down syndrome can be caused, at least in part, by a competition for the same miRNAs between HSA21 and non-HSA21 transcripts in early stages of the embryonic development. The main purpose of this project is to assess whether HSA21 mRNAs - that are overexpressed in DS embryos - can potentially function as miRNA "sponges", acting as ceRNAs and thus perturbing the expression of non-HSA21 mRNAs involved in the organogenesis.

The starting hypothesis is that the presence - throughout the entire embryonic development - of supernumerary copies of HSA21 mRNAs in DS embryos may disrupt the physiological mRNAs/miRNAs balance. It would affect the miRNA-mediated gene silencing that physiologically occurs during the embryonic development. This hypothesis would explain - at least partially - the
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multisystemic nature of the DS pathological outcomes, the heterogeneous phenotype of DS individuals as well as the global gene expression deregulation observed in distinct cell types and tissues isolated from DS individuals.

To address this aim, my PhD project first focused on the definition of a network of potential interactions between HSA21 and non-HSA21 genes mediated by miRNAs' binding during the early stages of human embryonic development. Such network is built on mRNAs and miRNAs expression data collected at specific weeks of the embryogenesis, and is based on a computational prediction analysis of miRNA binding sites within 3'UTRs of all the expressed genes. Then, the research project focused on the potential perturbation of such network - in DS embryos - induced by the presence of supernumerary copy of HSA21 genes. Afterwards, the computationally predicted mRNA/miRNAs interactions were evaluated using experimental in vitro approaches to validate the potential role of a selected HSA21 gene (i.e. HUNK) as sponge for miRNAs.

The aim of the project was also to establish a potential regulatory crosstalk between HSA21 genes and those belonging to development- and embryo-related pathways. The hypothesis is that such mRNAs may reciprocally regulate their expression through a ceRNA (or ceRNA-like) network.

As stated in the seminal work of Salmena and colleagues (2011), the aim of this project is to demonstrate that HSA21 genes overexpressed in DS embryos and some developmentally relevant mRNAs "talk to each other using microRNA response elements, as letters of a new language".
5 Methods

5.1 Data processing and bioinformatics analysis

5.1.1 Expression data sets description and processing
To assess gene expression for mRNAs and miRNAs during human embryogenesis, two previously published data sets were analysed. For mRNAs expression, a microarray genome-wide expression study carried out on human embryos during weeks 4-9 of development was analysed (Yi et al., 2010). Microarray technology involves the hybridization of mRNA molecules to probes immobilized in known locations on a chip. Fluorescence signal intensity of each spot is detected as an indirect measure of a specific gene expression. This study was performed on three RNA samples from whole embryo for each week using the Human Genome U133 Plus 2.0 Array (Affymetrix), which contains 50093 transcripts accounting for 38500 human genes. Normalized mRNA expression data were retrieved from the public functional genomics data repository Gene Expression Omnibus (GEO) at the National Center of Biotechnology Information (NCBI), through Geo Series accession number GSE15744. Custom MATLAB scripts were used to process data. Particularly, probe set IDs were associated to corresponding gene symbols using the HG-U133A_2 Affymetrix annotation file and mean values were calculated across genes. After filtering out mean values among replicates inferior than an arbitrary threshold of 49.5 signal intensity, an mRNA expression matrix was compiled for weeks 4-6 of human embryogenesis.

For miRNAs expression, a recently published miRNA microarray study was analysed (Lin et al., 2013). The miRNA expression study was performed on small RNA samples extracted from 3 embryos at week 4, 3 embryos at week 5 and 2 embryos at week 6 of development in dual-sample experiments, using µParaflo microfluidic chip (Atactic Technologies), which covers 835 miRNAs annotated in the Sanger miRBase database (release 10.1). Normalized miRNA expression data were retrieved from GEO through Geo Series accession
number GSE46795. MATLAB scripts were used to calculate mean values among replicates and to set an arbitrary threshold of 49.5 signal intensity in order to compile a matrix for miRNA expression during human embryogenesis. Using a custom MATLAB script, Pearson’s linear correlation was calculated between each mRNA-miRNA pair after logarithmic transformation of expression data and a 2D correlation matrix was compiled.

5.1.2 mRNA-miRNA interactions prediction
Fasta file corresponding to the 3'UTR sequences of 11080 genes expressed during weeks 4-6 of human development were downloaded from UCSC Table Browser (Karolchik et al., 2004). MREs in these sequences were predicted executing TargetScan 6.0 algorithm implemented in Perl language. Target prediction data were filtered out to remove miRNAs not expressed in weeks 4-6 of human embryogenesis. Then, a binary matrix with 11080 rows and 134 columns was compiled according to MREs prediction, and 0 and 1 were used to indicate the absence and presence of miRNA/mRNA interaction, respectively.

5.1.3 Data integration and biclustering analysis
TargetScan prediction information was integrated with the expression correlation data using element-by-element product of the two matrices in MATLAB. The integrated association matrix thus compiled underwent biclustering analysis. PLAID model biclustering was performed with the R “biclust” package (Turner et al., 2005). This algorithm is an improvement of original ‘Plaid Models for Gene Expression Data’ (Lazzeroni and Owen, 2002). The plaid model allows a gene to be in more than one cluster, or in none at all. It consists of a series of additive layers intended to capture the underlying structure of a gene expression matrix. Each layer is fitted to linear model \( y \sim m + a + b \), that estimates three parameters: \( m \) (constant for all elements in the bicluster), \( a \) (constant for all rows in the bicluster) and \( b \) (constant for all columns).

For the biclustering analysis, background layer was considered absent in the data matrix. Before a layer is added, it's statistical significance is compared
Methods

against 3 layers obtained by random, 15 and 10 iterations were performed to find starting values and each layer, respectively. The row and column release probabilities were set to 0.6 and the maximum number of layers to 50. The biclustering algorithm was iterated 100 times.

5.1.4 Differential gene expression in DS embryos

RNA-sequencing data sets from 5 normal and 4 DS chorionic villus samples were downloaded from GEO (accession n. GSE42144) in SRA format and then converted into FASTQ format using fastq-dump function included in the SRA Toolkit. Reads quality was evaluated through the FASTQC software. RNA-Seq reads were mapped against human reference transcriptome and then genome hg19 using TopHat v.2 software, allowing 3 segment mismatch. Gene counts and RPKM (Reads Per Kilobase of transcript per Million mapped reads) normalization were performed using RNASeqGUI R package (Russo and Angelini, 2014) and a threshold of 1 RPKM was used. Average RPKM values for each gene in each sample group (euploid and DS) were calculated. To identify differentially expressed genes in DS versus euploid conditions, fold change (FC) was calculated as the ratio of average RPKM value for DS/average RPKM value for euploid. Genes with a FC equal or greater than 1.5 FC were considered up-regulated in DS.

5.2 Generation of plasmid constructs

The full 3'UTR of HUNK gene (NM_014586) and the five partially overlapping 3'UTR fragments of the same 3'UTR were amplified from cDNA of MCF7 cell line by polymerase chain reaction (PCR). Specific PCR primers were designed using Oligo 4.0 software and synthesized by IDT (Table 1).

PCR reactions were carried out in 25 µL reaction volume, using 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen), 2.5 µL of 10X High Fidelity PCR Buffer, 1 µL of MgSO₄ (50 mM), 0.5 µL of dNTP mix (10 mM), 0.7 µL of each primer (8 µM) and 1 µL of cDNA (50 ng/µL). Amplification was performed in Bio-Rad thermal cycler under the following conditions: 94°C for 1 min; then 35 cycles of 94°C for 30 sec, annealing temperature (Ta) for 30 sec,
Methods

68°C for 6 min; and a final extension at 68°C for 30 min. Amplicons were analysed by 1.2% agarose gel electrophoresis and then underwent automated Sanger sequencing.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size (bp)</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUNK_3UTR_FULL</td>
<td>CAGCGGGGTTTGGGGTAT</td>
<td>GCTTCCTGTGCCATTTTA</td>
<td>4831</td>
<td>60</td>
</tr>
<tr>
<td>HUNK_3UTR_FR_1</td>
<td>CAGCGGGGGTTTGGGGTAT</td>
<td>TTGAAATACGATCGCAACAG</td>
<td>1033</td>
<td>60</td>
</tr>
<tr>
<td>HUNK_3UTR_FR_2</td>
<td>AGAGGGAAAAAGATGATTGTA</td>
<td>GGAAGTACCCCTGCTCTCA</td>
<td>1339</td>
<td>57</td>
</tr>
<tr>
<td>HUNK_3UTR_FR_3</td>
<td>CTGCTGGAACCCTGTAG</td>
<td>CTGGTTCCAGTCAGATGCTA</td>
<td>996</td>
<td>56</td>
</tr>
<tr>
<td>HUNK_3UTR_FR_4</td>
<td>TCCCTCAGACTGCT</td>
<td>GACATCCCCCTCTGACCAT</td>
<td>1153</td>
<td>56</td>
</tr>
<tr>
<td>HUNK_3UTR_FR_5</td>
<td>CTGCCCTAAAGAAGACACAGA</td>
<td>GCTTCCTGTGCACATTTTA</td>
<td>934</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 1: Primer pairs used for HUNK 3'UTR amplification

PCR products were cloned into pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen). Particularly, 4 µL of PCR product was mixed with 1 µL of vector and 1 µL of salt solution (1.2 M NaCl, 0.06 M MgCl₂) and incubated at room temperature for 20 min. Subsequently, cloning reaction mix was used to transform 50 µL chemically competent *E. coli* DH5α. Cells were incubated 30 min in ice and then heat-shocked 1 min at 42°C. After a 1 hour incubation in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) at 37°C in a shaking incubator (200 rpm), two different volumes (100 and 200 µL) of cells were spread on selective LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) plates containing 100 µg/mL ampicillin and incubated at 37°C overnight. The following day, 10 colonies were picked and cultured in 5 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 100 µg/mL ampicillin overnight. Plasmid DNA was isolated from bacterial cells with the ISOLATE II Plasmid Mini Kit (Bioline), according to manufacturers’ instructions. Isolated plasmids were digested with KpnI restriction enzyme to analyse insert presence and orientation. Plasmids with the correct insert were sequenced.
5.3 Cell culture and transfection

Human Embryonic Kidney 293 (HEK293) cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mmol/L glutamine (Gibco), 100 units/mL penicillin (Gibco), and 100 units/mL streptomycin (Gibco) for 4-5 passages after thawing. Cultures were maintained in a humidified atmosphere and 5% CO$_2$ at 37°C. Cells were dissociated using Trypsin-EDTA (0.05%), phenol red (Gibco) and seeded in six-wells plates at 5.0x10$^5$ cells/well in 2 mL of complete medium 18h before transfection to ensure 70-90% confluence on the day of transfection. Plasmid transient transfection was performed with Lipofectamine 3000 Reagent (Invitrogen), using a 1:3 DNA:lipid ratio, according to manufacturer’s instructions. Particularly, 2 µg of plasmid and 6 µL of Lipofectamine 3000 were each diluted in 125 µL Opti-MEM Reduced-Serum Medium (Gibco). 5 µL P3000 Reagent was added to diluted DNA. Then DNA mix was added to diluted Lipofectamine 3000 and incubated at room temperature for 5 minutes in order to allow DNA-lipid complex formation. Subsequently, 250 µL of the DNA-lipid complex was added dropwise to cells in serum- and antibiotic-free medium. The same quantity of empty pcDNA3.1 V5/His TOPO plasmid with the same lipid-to-DNA ratio was used as the control. Cells were incubated at 37°C in a humidified atmosphere with 5% CO$_2$. 6 hours after transfection, transfection mixture was replaced with complete medium. Transfected cells were harvested 24, 32 and 48 hours after transfection.

5.4 RNA extraction and Reverse Transcription

Total RNA was isolated from HEK293 cells using mirVana miRNA Isolation kit (Ambion), according to manufacturer’s instructions. This kit combines the advantages of organic extraction and solid-phase extraction to isolate RNA avoiding the loss of small RNAs. The sample is first lysed in a denaturing lysis solution, which stabilizes RNA and inactivates RNases. The lysate is then extracted with Acid-Phenol:Chloroform, which removes most of the other cellular components, leaving a semi-pure RNA sample. This is further purified
Methods

over a glass-fiber filter specifically formulated for miRNA retention and finally the RNA is eluted with a low ionic-strength solution.

RNA integrity and concentration were evaluated by gel electrophoresis and spectrophotometry (NanoDrop Technologies).

For mRNA analysis, reverse transcription was performed on total isolated RNA with SUPERSCRIPT II Reverse Transcriptase (Invitrogen). Reaction mix of 1 µg RNA, 1 µL Oligo(dT)_{12-18} (500 µg/mL) and 1 µL dNTP mix (10 mM each) in a final volume of 12 µL was heated at 65°C for 5 min and then chilled on ice. Subsequently, 4 µL 5X First-Strand Buffer, 2 µL DTT (0.1 M) and 1 µL RNase OUT (40 units/µL) were added to the reaction mix and incubated at 42°C for 2 min. After the addition of 1 µL of SuperScript II RT enzyme, the reaction mix was incubated at 42°C for 50 min and then the enzyme was inactivated heating the mixture at 70°C for 15 min.

For miRNA analysis, reverse transcription was performed on total isolated RNA with the miSCRIPT II RT Kit (Qiagen). Reverse-transcription master mix was prepared with 4 µL 5X miScript HiSpec Buffer, 2 µL 10X miScript Nucleics Mix, 2 µL miScript Reverse Transcriptase Mix and 1 µg of total RNA in a final volume of 20 µl. Samples were incubated at 37°C for 60 min and then at 95°C for 5 min to inactivate the Reverse Transcriptase Mix.

5.5 Quantitative Real-Time PCR

All quantitative Real-Time PCRs were performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad). For mRNA quantification, 10 µL final volume reaction mix was prepared using 1 µL cDNA from 1/5th of RT reaction with 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad) and 0.4 µL of each primer (10 µM). After an initial polymerase activation step at 95°C for 2 min, 40 2-steps cycles of amplification were run at 95°C for 5 sec and 60°C for 30 sec. Melt-curve analysis was performed from 65°C to 95°C with a 0.5°C increment and 5 sec/step to verify PCR product specificity. The housekeeping GAPDH and HPRT genes were used as reference genes for data normalization and
relative gene expression was measured with the $2^{\Delta\Delta Ct}$ method, comparing the Ct values of the samples of interest with the control.

Mature miRNA quantification was performed using miSCRIPT SYBR Green PCR Kit with miSCRIPT Primer Assay (Qiagen). Reaction mix was prepared with 12.5 µL 2X QuantiTect SYBR Green PCR Master Mix, 2.5 µL 10X miScript Universal Primer, 2.5 µL miRNA-specific 10X miScript Primer Assay and 1 µL cDNA from 1/10th of RT reaction in 25 µL final volume. After an initial activation step at 95°C for 15 min, 40 cycles of amplification were run at 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. Dissociation curve analysis was performed from 65°C to 95°C with a 0.5°C increment and 5 sec/step to assess the specificity of the reaction. Relative quantification was performed using the Ct comparison method, after data normalization with miScript PCR Controls for RNU6B snRNA and SNORD61 snoRNA.

Gene specific primers used for quantitative Real-Time amplification of mRNAs and miRNAs are listed in Table 2 and Table 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>GCCCCTGGTGGACAACATC</td>
<td>TCAGAGACAGCCAGGAGAAAT</td>
<td>152</td>
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<tr>
<td>CLIC5</td>
<td>ACCCGCCCTTCCCTGACCTT</td>
<td>TGCTGCTTGGTTATTTTGATG</td>
<td>184</td>
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<tr>
<td>EPHA5</td>
<td>GGACCTGGGATGGATTTGCTT</td>
<td>TGTTTTCTTTGATGTTTTTCTCC</td>
<td>297</td>
</tr>
<tr>
<td>ERBB4</td>
<td>GAGATAACCAGCATTGGACGAC</td>
<td>AGTTTTGTCCACAGAATAATGC</td>
<td>140</td>
</tr>
<tr>
<td>HIPK2</td>
<td>GGAAGAGTAACGACGACCACG</td>
<td>GTTCTCTTTGACACGCTTG</td>
<td>107</td>
</tr>
<tr>
<td>HUNK</td>
<td>CAGGCTCGCTTATGACACGCA</td>
<td>GGTGGCAGGGGGATGAGACT</td>
<td>172</td>
</tr>
<tr>
<td>MECP2</td>
<td>GATCAATCCCCAGGGAGAACGC</td>
<td>CCTCTCCCAGTGTTACCCTGAAG</td>
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<tr>
<td>ONECUT2</td>
<td>CCGCAGAGTTGGAAGTGGCG</td>
<td>CGGGGTTTTGCTTCTCTGTA</td>
<td>190</td>
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<tr>
<td>WNT5A</td>
<td>CGGCCAGGTGTTAATTGAAG</td>
<td>GCATGTGGTCTTCTACCAGAAG</td>
<td>164</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCTCTCGACCCACCAACT</td>
<td>CCATCACGCCACAGTTCC</td>
<td>149</td>
</tr>
<tr>
<td>HPRT</td>
<td>TGGCGTCTGTAGGAGACT</td>
<td>CCCATCTCTCTCTACACATC</td>
<td>156</td>
</tr>
</tbody>
</table>

Table 2: Primer pairs used for mRNA expression quantification by Real-Time PCR
Table 3: miRNA specific primers used in quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Forward specific primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17</td>
<td>Hs_miR-17_2</td>
</tr>
<tr>
<td>miR-20a</td>
<td>Hs_miR-20a_1</td>
</tr>
<tr>
<td>miR-20b</td>
<td>Hs_miR-20b_1</td>
</tr>
<tr>
<td>miR-128</td>
<td>Hs_miR-128_1</td>
</tr>
<tr>
<td>miR-200c</td>
<td>Hs_miR-200c_1</td>
</tr>
<tr>
<td>miR-205</td>
<td>Hs_miR-205_1</td>
</tr>
<tr>
<td>RNU 6</td>
<td>Hs_RNU6-2_11</td>
</tr>
<tr>
<td>SNORD 61</td>
<td>Hs_SNORD61_11</td>
</tr>
</tbody>
</table>
6 Results

6.1 Gene expression during human embryogenesis

Embryogenesis is the process of cell division and differentiation that occurs during the early stages of embryo development, when most organs began to form. Mammalian development requires the specification of over 200 unique cell types from a single totipotent cell (Boyer et al., 2005). Human embryogenesis covers the first eight weeks following fertilization and is governed by a precise genetic program. During early and late embryo development, a large number of molecules participate in embryo patterning and organization in a spatial and temporal manner to ensure the adequate formation of an organism. Transcription factors and small RNAs play a crucial role in several developmental processes (Pauli et al., 2011). Moreover, epigenetics changes, including histone modification, DNA methylation, and chromatin condensation, regulate specific genes during embryonic development, dictating cell fate decisions (Barber and Rastegar, 2010).

In this complex scenario, it is essential to understand how the global gene expression deregulation, due to the presence of an extra copy of chromosome 21, may contribute to the multisystemic nature of DS phenotypes during embryogenesis.

Since human embryos are difficult to access for research, publicly available expression datasets were downloaded and re-analysed in this PhD project to identify the genes expressed during human embryogenesis. Particularly, a Chinese research group investigated gene expression during the early stages of human development. They performed two independent genome-wide microarray studies to evaluate expression of both mRNAs and miRNAs in human embryos (Lin et al., 2013; Yi et al., 2010). Since miRNA expression data were available for weeks 4-6, expression data for mRNA were filtered out to remove weeks with no corresponded miRNA expression data (weeks 7-9).
Results

Analysis of the mRNA microarray data showed that 11080 genes are expressed during weeks 4-6 of development and their number is quite constant during these weeks (Figure 7).

![Figure 7: mRNA expression during weeks 4-6 of human development]

Notably, statistical over-representation test performed with Panther Classification system showed that the 11080 genes expressed during weeks 4-6 were involved in biological processes such as “cell cycle”, “developmental process”, “anatomical structure morphogenesis”, “system development” and “nervous system development” (Figure 8). Expressed genes were classified according to their expression into three main groups: a) genes with an increasing expression throughout weeks 4-6, b) genes with a decreasing expression, and c) genes with an oscillatory trend of expression during weeks 4-6 of embryogenesis (Figure 9).
Results

Figure 8: Statistical over-representation test of 11080 expressed genes during weeks 4-6 of development, according to Panther GO-Slim Biological Process

Figure 9: Top 15 mRNAs expression levels during weeks 4-6 of human development
Analysis of miRNA datasets revealed that 134 out of 835 miRNAs are expressed during weeks 4-6 (Figure 10). These data showed that most of miRNAs (about 84%) are not expressed in these weeks in which organogenesis occurs. Similarly to the mRNAs, we could classify expressed miRNAs into three categories: a) miRNAs with an increasing or b) decreasing expression throughout weeks 4-6 and c) miRNAs with an oscillatory trend of expression (Figure 11).

![Figure 10: miRNA expression during weeks 4-6 of human development](image)
Results

6.2 Identification of miRNA-mRNA regulatory network

Expression data for mRNA and miRNA were used to predict miRNA-mRNA interactions to define a regulatory network during early human development. Since miRNAs act as negative regulators of mRNAs, we were particularly interested in specific miRNAs with anti-correlation with the expression of its target mRNA. Thus, mRNA and miRNA expression data, following logarithmic transformations, underwent pairwise Pearson’s linear correlation analysis, in order to compile a 2D correlation matrix for all possible combinations of miRNA and mRNA expression (134 columns and 11080 rows). Pearson’s correlation coefficient is a degree of linear dependence between two variable and ranges from -1 to 1, where 1 indicates total positive correlation, 0 indicates the absence of correlation and -1 indicates a total negative correlation. Distribution of miRNA-mRNA correlation coefficients was represented by a bimodal curve with two distinct peaks (local maxima) at the extreme end of correlation values, indicating that we found a very high percentage of positive/negative miRNA-gene associations in our analysis (Figure 12).
Results

Figure 12: Correlation distribution curve before (green) and after (red) integration of prediction of miRNA-mRNA interactions

However, these positive/negative correlations did not imply direct miRNA-mRNA interactions. Indeed, it is known that miRNA-mRNA interactions are guided by sequence complementarity of the 5’ end of the miRNA, called the seed region, with the 3’UTR of target mRNA. MicroRNA-mRNA expression correlation matrix was integrated with data from the computational seed prediction in mRNA 3’UTR sequences of all expressed genes. Therefore, 3’UTR sequences of 11080 genes expressed during weeks 4-6 of human development were downloaded from UCSC Table Browser (Karolchik et al., 2004). MiRNA responsive elements in these sequences were predicted using TargetScan 6.0 algorithm implemented in Perl. Target prediction data was filtered out to remove miRNAs not expressed in weeks 4-6 of human embryogenesis. Then, a binary matrix with 11080 rows and 134 columns was compiled according to MREs prediction, where 0 and 1 indicate absence and presence of interaction, respectively. Such target prediction information was
Results

integrated with the expression correlation information using element-by-element product of the two matrices. In this way, an integrated association matrix was compiled, where any association - positive or negative - that was in conflict with predicted target site information was removed. Distribution of filtered miRNA-mRNA correlation coefficients was represented by a bimodal curve with two distinct peaks at the extreme end of correlation values lower than the peaks of the correlation coefficient distribution before filtering, indicating that most of miRNA-mRNA pairs showed no more correlation (Figure 12).

Subsequently, the analysis focused on the identification of a potential network of miRNA-mRNA interactions. In order to address this issue, a biclustering approach on the integrated association matrix was adopted. Biclustering allows simultaneously clustering rows and columns of a matrix, grouping a subset of rows that exhibit similar behaviour across a subset of columns, or vice versa. In this case, biclustering was used to find a subset of mRNAs whit anti-correlation to miRNAs, outlining a group of genes that can potentially compete for binding to specific miRNAs. For this analysis was used the Plaid model biclustering which is more flexible and allows a gene to be in more than one cluster, or in none at all. The biclustering algorithm was iterated 100 times and extracted biclusters were evaluated in order to find the one with the maximum number of genes and miRNAs with an opposing correlation. Such analysis revealed a bicluster of 210 genes and 29 miRNAs with anti-correlation, indicating a potential miRNA-mRNA regulatory network during early human embryogenesis (Figure 13 and Figure 14).
Results

Figure 13: Bubbleplot representing biclustering results
The analysis of distribution of miRNA-mRNA correlation coefficients for such bicluster showed a strong anti-correlation, as indicated by the bimodal curve with a highest peak centred near -1 value (Figure 15).
Gene Ontology analysis of genes from this bicluster revealed that 49 out of 210 are involved in embryonic development processes such as “regulation of embryonic development” (GO:0045995), “embryonic organ development” (GO:0048568), and “nervous system development” (GO:0007399). Moreover, hypergeometric test was performed to calculate statistical significance for over-representation of embryo-related genes (corrected p-value <0.05). This analysis suggested that perturbations of the defined regulatory network of miRNAs and mRNAs could - in proof of principle - determine the alteration of normal embryonic development (Figure 16).
6.3 Perturbation of miRNA-mRNA regulatory network during DS embryogenesis

Starting from the hypothesis that during embryogenesis in DS embryos, HSA21 genes may perturb the physiological miRNA-mRNA regulatory network, acting as sponges to sequester miRNAs, we found six HSA21 genes (ADAMTS5, AGPAT3, BACE2, ERG, HUNK, SLC5A3) in the selected miRNA-mRNA bicluster. However, as not all HSA21 genes are overexpressed in trisomic cells/tissue, we analyzed gene expression data in early DS embryo development. To this aim, RNA-Sequencing (RNA-Seq) datasets from 5 normal and 4 DS chorionic villi samples (Jin et al., 2013) were downloaded from GEO (accession number GSE42144) and analyzed, as described in the Methods Section.

Raw reads in SRA format were first converted into FASTQ, and then it was evaluated the quality through FASTQC software. Per base and per sequence quality graphs showed a good quality of reads (Figure 17 and Figure 18).
Results

Figure 17: Per base read quality

Figure 18: Per sequence read quality
Results

Subsequently, RNA-Seq reads were mapped against the human reference genome hg19 using TopHat software. Quantification of gene expression levels by reads' counting and RPKM normalization were performed using RNASeqGUI package in R language (Russo and Angelini, 2014). RPKM counts distribution was analysed to assess samples variability (Figure 19).

![RPKM BoxPlot](image)

**Figure 19: Box plot of RPKM counts distribution**

Similarities and differences among samples were highlighted performing a PCA (Principal Component Analysis). This analysis showed that euploid and DS samples were different enough to be considered two distinct groups, with the exception of sample EU2, which was discarded from further analyses (Figure 20).
Results

Figure 20: PCA plot of euploid and DS samples before (upper) and after (lower) removing EU2 sample (red arrow).
Results

Applying an arbitrary threshold of 1 RPKM, 14705 coding genes were detected as expressed in chorionic villi, 14044 of which were expressed in both conditions, 355 only in DS and 306 only in euploid samples. Differential expression analysis showed that 1221 genes were up-regulated in Down syndrome with 25 up-regulated HSA21 genes. Two out of six HSA21 genes - i.e. HUNK and BACE2 - are overexpressed in DS embryos and belong to the previously described bicluster of miRNA-mRNAs. Such network consists of 210 potential ceRNAs and 29 miRNAs; however, not every mRNA is necessarily targeted by every miRNA. Indeed, ceRNA cross-regulation increases with the number of shared miRNAs and is weakened when a ceRNA pair is targeted by too many non-shared miRNAs. Thus, the number of MREs and the anti-correlation values of HUNK and BACE2 genes with the miRNAs included in the selected bicluster were analysed. HUNK and BACE2 genes were both predicted to interact with 19 out of 29 miRNAs contained in this bicluster. However, HUNK gene showed more negative anti-correlation values, and therefore it was selected for further in vitro studies.

6.4 The role of HUNK as a ceRNA

HUNK (hormonally up-regulated Neu-associated kinase) gene spans 171318 bp on the forward strand of human chromosome 21q22 and consists of 11 exons. HUNK is a sucrose non-fermenting 1 (Snf-1)/AMPK family of serine/threonine protein kinases member, that was first identified in the murine mammary gland (Gardner et al., 2000). AMPK family regulates cellular metabolism, stress responses and several processes relevant to tumorigenesis, including proliferation, differentiation, survival, and migration (Yeh et al., 2011). Consistently, Hunk is overexpressed in several human cancers and is involved in the onset and the metastasis of specific human mammary tumors (Yeh et al., 2013). In situ hybridization showed that Hunk expression is developmentally regulated and tissue-specific in fetal mice and restricted to subsets of cells in the adult mouse (Gardner et al., 2000). Despite these findings suggest a role for Hunk in murine development, Hunk deletion in mice did not alter viability,
Results

fertility, longevity, revealing HUNK is dispensable for normal development (Wertheim et al., 2009).

The ability of HUNK to act as ceRNA during human embryogenesis was investigated in the course of this project overexpressing HUNK 3'UTR in HEK293 cell model. HEK293 cells were used since these cells are very easy to grow in culture and transfet very readily. Moreover, gene expression data from RNA-Seq experiments performed in our laboratory and from The Human Protein Atlas database indicated that HEK293 share 80% genes with embryonic samples.

The 3'UTR is transcribed, but it is not translated into protein; however it can exert a regulatory function on gene expression through miRNA binding. HUNK 3'UTR (4880 bp) was amplified by RT-PCR, cloned into expression plasmid pcDNA3.1 V5/His TOPO, and used to transfet HEK293 cell line. Empty pcDNA3.1 V5/His TOPO vector was used as control. According to the results of our computational analysis of the regulatory network, and in line with the ceRNA mechanism, HUNK 3'UTR overexpression should perturb the distribution of miRNAs on their targets, acting as a decoy for specific miRNAs. Consequently, as miRNAs are able to repress specific mRNAs, altering the expression level of a single ceRNA may have significant effect on other ceRNAs with shared MREs. Thus, we evaluated the expression of genes belonging to the ceRNA network 24, 30 and 48 hours after HUNK 3'UTR transfection by quantitative Real-Time PCR. First, endogenous HUNK gene expression was evaluated with specific primer pairs in the coding exons 10 and 11. Results showed that overexpression of HUNK 3'UTR de-repressed HUNK transcript itself, inducing an overexpression of its endogenous transcript. Particularly, HUNK expression increased with increasing time after transfection (Figure 21).
Then, the potential role of *HUNK* gene overexpression in the perturbation of embryogenesis in DS was further investigated. First, genes included in the selected bicluster were analysed in order to identify candidate genes involved in human embryogenesis. As previously cited, 49 out of 210 genes of the selected bicluster resulted to play a role in human embryogenesis, according to gene ontology (GO:0048568, GO:0045995, GO:0007399). Then, a comparative analysis with the genes differentially expressed in DS embryos was performed to assess whether some of these 49 embryo-related genes were up-regulated during DS embryogenesis. Hence, 8 embryo-related genes (*BCL2, CLIC5, EPHA5, ERBB4, HIPK2, MECP2, ONECUT2, and WNT5A*), included in the bicluster - and up-regulated in DS embryos - were selected, and their expression was evaluated in *HUNK* 3'UTR transfected samples (Figure 22).
Overexpression of *HUNK* 3'UTR determined an increase of *BCL2*, *CLIC5*, *EPHA5*, *ERBB4*, *HIPK2*, *MECP2*, *ONECUT2*, and *WNT5A* genes expression levels, confirming the computational analysis used to define the miRNA/mRNA network.

Since *HUNK* 3'UTR may act as decoy for the miRNAs belonging to this ceRNA network, the expression of 6 selected miRNAs (miR-17, miR-20a, miR-20b, miR-128, miR-200c, and miR-205) was investigated. To this end, quantitative Real-Time PCR was performed in transfected cells 24 and 30 hours after transfection (Figure 23).
Results

Notably, in line with our hypothesis, all miRNAs but miR-205 showed a decrease of expression, suggesting they are sequestered by the 3'UTR of _HUNK_ gene.

Then, to test whether the ceRNA effect of _HUNK_ gene was due to a specific region of its 3'UTR, and in turn to specific MREs, _HUNK_ 3'UTR was divided into 5 overlapping fragments. Each fragment was amplified, cloned into pcDNA3.1 V5/HIS TOPO vector and used to transfect HEK293 cells. Expression levels of _BCL2_, _CLIC5_, _HIPK2_, _MECP2_, _ONECUT2_, and _WNT5A_ genes was analysed by quantitative Real-Time PCR (Figure 24).
Results

Moreover, the expression of the six selected miRNAs was analysed through quantitative Real-Time PCR in cells transfected with partial HUNK 3’UTR fragments (Figure 25).
Results

Figure 25: Expression analysis of selected miRNAs 24h after transfection of partial HUNK 3'UTR fragments
7 Discussion

Down syndrome (DS) is the most frequent human aneuploidy, caused by an extra copy of all or part of human chromosome 21 (HSA21). DS is a complex genetic condition characterized by over 80 clinically different phenotypes of variable penetrance and expressivity. Individuals with DS show alterations - both structural and functional - affecting distinct organs and systems, suggesting a crucial role of Trisomy 21 in determining alterations of developmental processes. Thus, overexpression of individual genes cannot be considered independently when one considers phenotype-genotype correlations. Indeed, global gene deregulation suggests a more complex mechanism of gene expression regulation, which involves direct and/or indirect interactions among HSA21 gene products and genes located on the other chromosomes.

This PhD project investigated the potential role of HSA21 genes - that are overexpressed in DS embryos - as competing endogenous RNA (ceRNA) during embryogenesis in foetuses with trisomy 21. The hypothesis is that these genes may act as miRNA "sponges", perturbing the expression of non-HSA21 mRNAs involved in the organogenesis. The supernumerary copies of HSA21 mRNAs would - in principle - disrupt the physiological mRNAs/miRNAs balance in DS embryos, thus affecting the miRNA-mediated gene silencing network that physiologically occurs during embryonic development.

Using bioinformatics approaches and in vitro experimental validation, a potential regulatory network between miRNA and mRNA has been defined during the early stages of human development. It has been demonstrated that some HSA21 genes can crosstalk with other mRNAs through a competition for common miRNAs. Particularly, the results demonstrate that the 3'UTR of HUNK gene acts as decoy for miR-17, miR-20a, miR-20b, miR-128, and miR-200c. This "sponge" effect determines an increase of BCL2, CLIC5, EPHA5, ERBB4, HIPK2, MECP2, ONECUT2, and WNT5A expression levels.
Discussion

Interestingly, MECP2 is essential for embryonic development. Its overexpression in mouse embryos leads to motor coordination deficit, impairment in learning and memory, hypotonia, severe mental retardation, recurrent infections. MECP2 encodes for methyl-CpG-binding protein 2 (MeCP2), which binds specifically to methylated DNA and is able to repress transcription from methylated gene promoters. WNT5A (Wingless-Type MMTV Integration Site Family, Member 5A) gene is implicated in several developmental processes, including regulation of cell fate and patterning during embryogenesis. Its overexpression leads to severe outgrowth defects, including gastrointestinal, limbs, facial structures defects, and mental impairment. MECP2 and WNT5A overexpression in foetuses with DS are in agreement with the potential defects occurring during embryogenesis in DS. The results described in this project show that the multisystem alterations of Down syndrome may be caused, at least partly, by a competition between HSA21 and non-HSA21 transcripts for the same miRNAs. Such event is likely to occur in early stages of the embryonic development. These findings shed new light on HSA21 genes. Indeed, if data will be supported by further experimental evidences (e.g. cellular models of differentiation or animal models), it will confer a previously unrecognised role - encrypted in the mRNA itself and not linked to the protein-coding potential - to HSA21 genes in Down syndrome. This project has highlighted for the first time the existence of a crosstalk among mRNAs during human embryogenesis, demonstrating that HSA21 genes - that are overexpressed in DS embryos - and some developmentally relevant mRNAs "talk to each other using microRNA response elements, as letters of a new language". Concernedly, beyond their functioning as cis regulatory elements, UTRs can act as trans modulator of gene expression through miRNA binding, allowing RNA to communicate each other through the letters of the "ceRNA language".

This new regulatory layer, based on mRNA-miRNA interaction network, provides new perspectives to the study of human embryogenesis regulation and to the deregulation of key mechanisms of pathogenesis. Furthermore, it might
Discussion

account for a portion of missing genetic and epigenetic variability in the etiology of diseases, particularly of DS. Thus, a novel dimension in gene regulation, based on cellular mRNAs and miRNAs homeostasis, is proposed herein. The disruption of this balance - caused for instance by HSA21 gene triplication - would affect crosstalk among ceRNAs, promoting genetic syndromes (like DS) due to aneuploidy or other similar genetic conditions linked to large chromosomal rearrangements.

However, it remains to be established whether other HSA21 genes besides HUNK may exert a similar role of ceRNA during embryogenesis. In addition, animal model studies of embryogenesis and DS will be needed to clarify the regulatory network of miRNA-mRNA interactions during human development and the effect of perturbations of such network on phenotype manifestations.
References

8 References


