Haematopoietic development and leukaemia in Down syndrome

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Abstract

Children with constitutional trisomy 21 (cT21, Down Syndrome, DS) are at a higher risk for both myeloid and B-lymphoid leukaemias. The myeloid leukaemias are often preceded by a transient neonatal pre-leukaemic syndrome, Transient Abnormal Myelopoiesis (TAM). TAM is caused by cooperation between cT21 and acquired somatic N-terminal truncating mutations in the key haematopoietic transcription factor *GATA1.* These mutations, which are not leukaemogenic in the absence of cT21, are found in almost one third of neonates with DS. Analysis of primary human fetal liver haematopoietic cells and of human embryonic stem cells demonstrates that cT21 itself substantially alters human fetal haematopoietic development. Consequently many haematopoietic developmental defects are observed in neonates with DS even in the absence of TAM. Although studies in mouse models have suggested a pathogenic role of deregulated expression of several chromosome 21-encoded genes, their role in human leukaemogenesis remains unclear. As cT21 exists in all embryonic cells, the molecular basis of cT21 associated leukaemias likely reflects a complex interaction between deregulated gene expression in haematopoietic cells and the fetal hematopoietic microenvironment in DS.

Introduction

Children with constitutional trisomy 21 (cT21, Down syndrome, DS) have a remarkably high risk of acute leukaemia (Hasle et al, 2000). The incidence of acute myeloid leukaemia (known as ML-DS) is ~150 times greater in young children with DS compared to children of the same age without DS while the incidence of acute B-cell lymphoblastic leukaemia (B-ALL) is ~33 times higher (Hasle et al, 2000; Zipursky, 2000). The unique features of DS-associated leukaemias point to the crucial role played by cT21 in their pathogenesis and provide tractable models of human leukaemogenesis (Malinge et al, 2009; Roy et al, 2009; Izraeli et al, 2014). Here we discuss recent insights into the natural history and pathogenesis of acute leukaemia in children with DS with the major emphasis on the perturbation of fetal and postnatal haematopoietic development by T21, that may render haematopoietic stem and progenitor cells (HSPC) more susceptible to leukaemic transformation.

DS-associated leukaemias – an overview

As we wish to focus here on the aspects of perinatal haematopoiesis that may predispose children with DS to leukaemia, we will only summarize the main features of the leukaemias of DS. For more detailed description of the clinical features of these leukaemias several recent reviews are recommended (Buitenkamp*, et al* 2014, Izraeli*, et al* 2014, Malinge*, et al* 2009, O'Brien*, et al* 2013, Taga*, et al* 2012, Zwaan*, et al* 2008).

DS-associated Acute Lymphoid Leukaemia

The lymphoid leukaemias of DS (DS-ALL) are almost exclusively B precursor ALLs. In a recent large series of DS-ALLs there were only 5 cases of T-ALL among 700 patients (Buitenkamp*, et al* 2014). Also, in a sharp contrast to the myeloid neoplasms, they almost never occur in infants. Clinically, the outcome of DS-ALL is significantly worse than the sporadic childhood B cell precursor ALLs because of intrinsic resistance to therapy and increased treatment related mortality (Buitenkamp*, et al* 2014, Izraeli*, et al* 2014).

While all known cytogenetic subgroups of childhood B ALLs can be found, the common abnormalities, ETV6-RUNX1 fusion and hyperdiploidy, are less frequent in DS (Buitenkamp*, et al* 2014, Forestier*, et al* 2008). Up to 60% of DS-ALLs have aberrant expression of the cytokine receptor CRLF2 that is often associated with additional mutations activating JAK-STAT growth-promoting signalling (Bercovich*, et al* 2008, Hertzberg*, et al* 2010, Mullighan*, et al* 2009, Russell*, et al* 2009, Shochat*, et al* 2011, Tal*, et al* 2014). The aberrant expression of this receptor is caused by genomic rearrangements consisting either of a translocation into the IgH locus control region or a micro-deletion upstream to the *CRLF2* gene, located on the pseudoautosomal part of the sex chromosomes. This deletion fuses *CRLF2* with the promoter of an upstream constitutively expressed *P2RY8* gene. Analysis of the breakpoint sequences suggest that this rearrangement is mediated by *RAG1* or *RAG2* in early B cell precursors (Mullighan*, et al* 2009).

DS-associated Acute Myeloid Leukaemia (AML)

The myeloid leukaemias of Down Syndrome were given a special WHO sub-classification (ML-DS) because of their unique clinical and biological features (Hasle*, et al* 2003, Vardiman*, et al* 2009). Immunophenotypically these are erythro-megakaryoblastic leukaemias (Langebrake*, et al* 2005, Zwaan*, et al* 2008). They are diagnosed before the age of 5 years and often present with unexplained thrombocytopenia and/or myelodysplasia. Most evidence indicates that ML-DS is always preceded by the neonatal pre-leukaemic syndrome Transient Abnormal Myelopoiesis (TAM) that may, or may not, be clinically apparent. Unlike acute megakaryoblasitc leukaemias in non-DS patients, they usually respond well to therapy with most patients cured. The mechanism of this exquisite sensitivity to chemotherapy, in particular to cytosine arabinoside (Ara-C), is unclear although many hypotheses have been suggested (Edwards*, et al* 2009, Ge*, et al* 2004, Ge*, et al* 2005).

Genetically ML-DS are characterized by an acquired mutation in the *GATA1* gene that will be discussed in detail below. The mutation in *GATA1* is necessary but insufficient for development of ML-DS. Recent exome sequencing studies of ML-DS revealed a high frequency of mutations in cohesins, CTCF or other chromatin regulators (Nikolaev et al, 2013; Yoshida et al, 2013). . While the normal function of these proteins is incompletely understood, they are believed mainly to organize major areas of transcription along and between chromosomes. Why these mutations are especially common in ML-DS is unclear but they may be related to the presence of trisomy. The other type of mutations, believed to enhance growth and proliferation, are in common signalling pathways such as RAS and the

thrombopoietin receptor MPL or downstream JAK-STAT signalling (Nikolaev*, et al* 2013, Yoshida*, et al* 2013). Importantly genomic analysis of ML-DS confirmed that it evolves from the cells responsible for TAM (also known as Transient Myeloproliferative Disorder).

A clinical pre-leukaemic syndrome: Transient Abnormal Myelopoiesis (TAM)

While it is clear that most childhood leukaemias arise from molecularly identifiable pre-leukaemic cells(Greaves 2003), in children with DS the pre-leukaemic syndrome can be clinically diagnosed. Most children who present with ML-DS have a history of overt TAM in the neonatal period (Klusmann et al, 2008). Conventionally TAM has been defined by its clinical and haematological features as a transient, clonal, neonatal myeloproliferative disorder unique to DS (Zipursky, 2003; Malinge et al, 2009). The defining feature used in the WHO classification (Vardiman et al; 2009), and most studies, is the presence of increased peripheral blood blast cells (Klusmann et al, 2008; Gamis et al, 2011), although we now know that this definition is too non-specific since virtually all neonates with DS have increased peripheral blood blast cells (Roberts et al, 2013). While a high percentage of blast cells (>20%) remains a good guide to a probable diagnosis of TAM, the most specific diagnostic parameter for TAM is the presence of an acquired mutation in exon 2 or 3 of the *GATA1* gene (Rainis et al, 2003; Hitzler et al, 2003; Groet et al, 2003; Ahmed et al, 2004; Malinge et al, 2009; Roberts et al, 2013; Yoshida et al, 2013). While in many cases, neonates with a *GATA1* mutation have clinical and haematological features consistent with classical descriptions

of TAM, we have recently suggested the term 'silent TAM' for the equally common scenario of DS neonates who have a *GATA1* mutation, usually because the size of the mutant *GATA1* clone is very small (Roberts et al, 2013).

Epidemiology: Estimates of the frequency of TAM vary from <5% to 30% of neonates with DS depending on the diagnostic criteria used and the study design. The lowest frequency (3.8%) was found in a large systematic *GATA1* mutation screen by Sanger sequencing of PCR products (Pine et al, 2007). This low frequency likely reflects the relatively insensitive methodology as the recent prospective study in 200 neonates with DS found a frequency of *GATA1* mutations of 8.5%, using a combination of standard Sanger sequencing with direct high performance liquid chromatography (dHPLC), which matches the 5-10% prevalence of TAM diagnosed by clinical and haematological criteria in most studies (Malinge et al, 2009). Interestingly, very sensitive next generation sequencing (NGS) methodology, which allows very small mutant *GATA1* clones to be detected ($\geq$0.3%), identified *GATA1* mutations in 30% of all neonates with DS, at least half of which are 'silent' (Roberts et al, 2013; discussed below).

Clinical features of TAM (Table 1): Classical TAM has a variable clinical presentation. Occasionally TAM presents in fetal life when it may lead to intrauterine or neonatal death (Heald et al, 2007; Klusmann et al, 2008; Gamis et al, 2011). More often TAM presents in the first few days of life, where the presentation varies from asymptomatic alterations in the blood count and/or blood film to disseminated leukaemic infiltration. Clinically severe TAM (liver

failure/fibrosis, ascites, pleural/pericardial effusion, renal failure and/or coagulopathy) affects 10-30% of patients with clinically diagnosed TAM (Massey et al, 2006; Klusmann et al, 2008; Gamis et al, 2011; Roberts et al, 2013).

Haematological features of TAM (Table 2): The most common haematological findings are a raised leucocyte count with increased peripheral blood blast cells, neutrophils, basophils and myelocytes (Roberts et al, 2013). Some neonates with TAM also have eosinophilia (Maroz et al, 2013). There is no difference in platelet count between DS neonates with and without TAM and very few neonates with TAM are anaemic (Gamis et al, 2011; Roberts et al, 2013). Importantly, no haematological features are specific for TAM except for large numbers of circulating blasts (Figure 1). Although there is no specific blast % threshold which reliably identifies all cases of TAM in DS neonates, we have found that the presence of blasts >20% on a peripheral blood film is invariably associated with a *GATA1* mutation and hence a diagnosis of TAM. By contrast, the significance of blasts <20% in a neonate with DS can only be assessed by carrying out *GATA1* mutation analysis.

Silent TAM: Around 20% of neonates with DS have *GATA1* mutations that are clinically and haematologically silent ('silent TAM'). These neonates have no features which differentiate them from DS neonates who have no *GATA1* mutations. Notably, neonates with DS have a high frequency of jaundice and thrombocytopenia even in the absence of *GATA1* mutations. Usually, but not

always, the small mutant *GATA1* clones found in 'silent TAM' can only detected using very sensitive methods, such as NGS (Roberts et al, 2013).

Natural history of TAM: Retrospective clinical studies show that most cases of classical TAM spontaneously resolve within a few week or months of birth (Klusmann et al, 2008; Gamis et al, 2011). A small proportion of babies with TAM will die from their disease, usually due to liver failure caused by hepatic fibrosis and blast cell infiltration (Klusmann et al, 2008; Gamis et al, 2011). Previous studies showing a mortality rate of ~20% in TAM are likely to be an over-estimate since the diagnosis of TAM was based on clinical and haematological criteria and milder or asymptomatic cases were likely to have been missed. Estimates of the risk of ML-DS following TAM vary from 5% in the recent prospective study (Roberts et al, 2013) to 30% in retrospective studies of clinically diagnosed TAM (Lange et al, 1998; Massey et al, 2006; Klusmann et al, 2008; Gamis et al, 2011). No specific clinical, haematological or molecular features predict the risk of transformation to ML-DS. A fuller picture of the natural history of TAM will require prospective studies of children with DS with and without *GATA1* mutations up to the age of 5 years (beyond this age, ML-DS is exceptionally rare (Hasle et al, 2008).

Diagnosis and monitoring of neonates with TAM: Since DS neonates with a *GATA1* mutation (both clinically overt TAM and 'silent TAM') may develop ML-DS before the age of 5 years, the best way to identify all those at risk is to screen all neonates with DS for *GATA1* mutations using a combination of standard (eg direct Sanger sequencing and DHPLC) and more sensitive

techniques (eg NGS). *GATA1* mutation analysis may also be useful in the acute setting to identify atypical cases of TAM, eg where the presentation is predominantly one of liver involvement with non-specific haematological features. The role of serial monitoring of the mutant *GATA1* clone in neonates with TAM is unclear and is currently being investigated.

Molecular pathogenesis of TAM: We, and others, have shown that virtually all cases of TAM and ML-DS have N-terminal truncating *GATA1* mutations (Wechsler et al, 2002; Hitzler et al, 2003; Rainis et al, 2003; Groet et al, 2003; Xu et al, 2003; Ahmed et al, 2004; Alford et al, 2011). *GATA1* mutations are present at birth both in DS neonates with TAM and, through retrospective analysis of neonatal blood spots, also in children with ML-DS without a previous history of TAM (Ahmed et al, 2004). It is not yet clear at what stage in fetal development *GATA1* mutations arise since the earliest point in gestation at which mutations have been identified is 21 weeks (Taub et al, 2004). *GATA1* mutations disappear when TAM (or ML-DS) enters remission indicating that these are acquired events (Ahmed et al, 2004; Yoshida et al, 2013). Since N-terminal truncating *GATA1* mutations are not leukaemogenic in the absence of cT21 (Hollanda et al, 2006) and do not appear to cause leukaemia in children with DS over the age of 5 years (Hasle et al, 2008), this strongly implicates unique features of cT21 fetal haematopoiesis in the transforming activity of mutant *GATA1*.

The reason(s) for the high frequency of *GATA1* mutations in neonates with DS are not known. It is notable that *GATA1* mutations are not only found in ~30% of all DS neonates but also that in 10-20% of these cases there are multiple

mutant *GATA1* clones (Alford et al, 2011; Roberts et al, 2013). It seems likely that the N-terminal truncated (short) GATA1 protein (GATA1s) confers a selective growth advantage to fetal haematopoietic cells harbouring mutant *GATA1*-containing clones. Alternatively, or in addition, trisomic cells may have a 'mutator phenotype' due to chromosomal instability. Evidence for a role of chromosomal instability in DS malignancies is conflicting (Ganmore et al, 2009; Nizetic and de Groet, 2012). The low frequency of most types of solid tumour in DS (Hasle et al, 2000) suggests that, in contrast to acquired aneuploidies, cT21 is not generally associated with a 'mutator phenotype' although it is possible that cT21 induces high mutation rates only at specific genomic loci (e.g. *GATA1*), as in the kataegis phenotype (Nik-Zainal et al, 2012).

Most of the mutations (97%) are found in exon 2 of the *GATA1* gene, including insertions, deletions and point mutations, the remainder occurring in exon 3.1 (Alford et al, 2011). All mutations lead to expression of a truncated GATA1s protein (Wechsler et al, 2002; Rainis et al, 2003). The type of mutation does not predict which patients with TAM will later progress to ML-DS (Alford et al, 2011). Indeed, whole exome/genome sequencing of paired TAM and ML-DS samples indicates that ML-DS may develop not only from major *GATA1* sub-clones present in the TAM phase of the disease but also from minor mutant *GATA1* clones (Yoshida et al, 2013). This approach has also shown that TAM samples contain very few somatic mutations (mean $\leq$2) compared to other cancers (Yoshida et al, 2013; Nikolaev et al, 2013). Thus cT21 and mutated *GATA1* are both necessary and sufficient for generation of TAM.

How GATA1s contributes to the TAM phenotype is unclear. Originally it was believed that the loss of this “trans-activation” domain reduces the activity of

GATA1 in regulating the terminal differentiation of megakaryocytes, thereby leading to accumulation of poorly differentiated megakaryocytic progenitors (Wechsler, *et al* 2002). Yet it is highly interesting that inherited mutations in the zinc fingers of *GATA1* that cause anaemia and thrombocytopenia due to a block in megakaryocyte-erythroid differentiation, are not found in TAM or ML-DS (Nichols, *et al* 2000). Rare patients with inherited GATA1s mutations and anemia have been recently reported. These patients do not develop leukemia (Hollanda, *et al* 2006, Klar, *et al* 2014, Sankaran, *et al* 2012). This suggests that both the DNA binding and protein interaction zinc finger domains, preserved in GATA1s, and cT21 are necessary for leukemic transformation. Furthermore forced expression of GATA1s in fetal liver haematopoietic progenitors from Gata1 wild type (wt) mice causes marked expansion of megakayoblastic progenitors, supporting a gain of function mechanism (Salek-Ardakani, *et al* 2009, Toki, *et al* 2013). Klusmann et al suggested that GATA1s enhances fetal haematopoietic cell proliferation through relieving the suppressive effect of GATA1 on E2F1 and collaboration with the insulin-like growth factor (IGF) signalling pathway (Klusmann, *et al* 2010a). Supporting this hypothesis is the recent report of a mutated *GATA1* lacking just the E2F1 interaction domain in the amino terminus of *GATA1* (Toki, *et al* 2013).

Abnormal fetal haematopoiesis in Down syndrome

Most evidence indicates that *GATA1* mutations arise exclusively in fetal liver HSPC. TAM, where it is clinically overt, manifests with involvement of the liver rather than bone marrow (Taub et al, 2004; Heald et al, 2007; Klusmann et al, 2008; Gamis et al, 2011). Acquisition of *GATA1* mutations selectively in fetal

liver HSPC is, arguably, the most logical explanation both for the spontaneous remission of the majority of *GATA1* mutant clones within the first few months of life and for the rarity of leukaemia due to *GATA1* mutations in children with DS after their 5th birthday. Similarly, there is no good evidence that TAM can be initiated after the first few months of life, presumably because the relevant 'susceptible' HSPC populations, and/or supportive microenvironment, are no longer present. This correlative evidence from humans for the exclusive function of GATA1s mutants in the fetal liver environment is further strengthened by the analysis of Gata1s knock-in mice (Li*, et al* 2005). These mice display a transient wave of fetal megakaryoblastic proliferation without apparent postnatal haematological abnormalities.

Increased megakaryocyte-erythroid progenitors in DS fetal liver: It is now clear from studies in primary human fetal liver cells (Tunstall-Pedoe et al, 2008; Chou et al, 2008; Roy et al, 2012), human embryonic stem cells (hESC) and trisomic induced pluripotent stem cells (iPSC) (MacLean et al, 2012; Chou et al, 2012) that the presence of cT21 alters the balance of HSPC differentiation. Notably, megakaryocyte-erythroid progenitors (MEP) and megakaryocytes (Figure 2) are increased in cT21 fetal liver and exhibit enhanced proliferative properties *in vitro* compared to disomic cells (Roy et al, 2012). These data support the contention that cT21 itself promotes abnormal megakaryocyte-erythroid expansion of fetal liver cells and that the somatic mutation of *GATA1* transforms these progenitors to generate clonal congenital transient leukaemia or TAM (Izraeli 2008).

Increased numbers of megakaryocyte-erythroid biased HSC in DS fetal liver: To investigate whether the abnormalities in DS fetal liver are confined to committed myeloid progenitors (MEP and CMP) or extend to include the HSC and/or multipotential progenitor (MPP) compartment, we recently performed detailed immunophenotypic and functional analysis of the HSC/MPP, committed myeloid progenitor and B-lymphoid compartments of human cT21 fetal liver without *GATA1* mutations and compared these with normal human fetal liver (Roy et al, 2012). This showed that in DS, immunophenotypically-defined HSC (CD34+CD38-CD90+CD45RA+) are increased in fetal liver and that *in vitro* purified HSC display an erythroid-megakaryocyte biased gene expression signature and generate much larger numbers of megakaryocyte and erythroid lineage progenitor cells than normal fetal liver HSC (Roy et al, 2012). Whether cT21-driven proliferation of megakaryocyte-biased' HSC and/or progenitor cells increases the likelihood of acquiring *GATA1* mutations or simply leads to a survival advantage of cells which acquire such mutations is still unclear. However, all available evidence indicates that cT21-mediated perturbation of fetal liver haematopoiesis is an essential prerequisite for the leukaemogenic properties of N-terminal truncating *GATA1* mutations.

Perturbation of B cell development in DS fetal liver: Since there is no overall increase in the HSCP compartment in DS fetal liver, this suggested that megakaryocyte-erythroid expansion might compromise the development of other lineages. Indeed, we found reduced numbers of both granulocyte-monocyte progenitors (GMP) and B-cell progenitors (BCP) in second trimester fetal liver (Roy et al, 2012). B-lymphoid development was severely impaired

with ~10-fold reduction in pre-pro BCP as well as markedly reduced B-cell potential of HSC in tandem with reduced HSC lymphoid gene expression priming. We speculate that perturbation of fetal B-cell development in DS may underlie both the immune deficiency common in children with DS (Kusters et al, 2009) and also the increased susceptibility to B-ALL (Zipursky, 2000; Whitlock et al, 2005)(Buitenkamp*, et al* 2014). It is possible that a peri- or post-natal compensatory drive to B-lymphopoiesis increases the likelihood of acquiring leukaemogenic mutations in young children with DS.

Studies of haematopoiesis in hESC and iPSC: MacLean et al recently showed that cT21 hESC and iPSC differentiated under fetal liver-like conditions generate increased erythroid and megakaryocyte colony-forming cells compared to isogenic disomic clones (MacLean et al, 2012) supporting the findings in primary human fetal liver cells (Roy et al, 2012). Interestingly, with the aim of modelling yolk sac haematopoiesis, Chou et al, differentiated cT21 iPSC under broadly similar conditions and showed enhanced erythropoiesis but megakaryocyte production was normal and myelopoiesis was reduced (Chou et al, 2012). These data suggest that the effects of cT21 might be developmental stage specific. Whether this has an impact on the timing of acquisition of *GATA1* mutations and their functional consequences remains to be determined.

Role of the microenvironment in abnormal fetal haematopoiesis in DS: Although, clinical and biological evidence suggests that fetal liver is likely to provide the specialised microenvironment necessary for driving and/or

maintaining abnormal haematopoiesis in DS, the factors responsible for perturbing haematopoiesis in DS are not known. Similarly, it is not clear whether cT21 influences the support function of the fetal liver microenvironment. There is some evidence that altered responsiveness to IGFs might play a role. In the mouse, fetal HSC expansion is supported by IGF2 produced by unique fetal liver stromal cells in contrast to adult bone marrow HSPC which depend on osteoblast-derived IGF1 (Zhang and Lodish, 2004; Garrett and Emerson, 2008; Chou and Lodish, 2010). Similarly, murine fetal, but not adult, megakaryocyte progenitors, are dependent for their survival and proliferation on the IGF signalling pathway, as are ML-DS and TAM cells (Klusmann et al, 2010a). Thus, developmentally-regulated IGF signalling mediated by cells of the fetal liver microenvironment may contribute to the HSC megakaryocyte-erythroid bias and MEP expansion in DS fetal liver, although how this is linked to cT21 is unclear.

Post-natal haematopoiesis in Down syndrome

Haematological abnormalities in neonates with DS: Retrospective studies have reported that haematological abnormalities occur more frequently in neonates with DS (Henry*, et al* 2007, Kivivuori*, et al* 1996, Starc 1992), raising the possibility that cT21 may continue to exert effects on haematopoiesis in post-natal life. Consistent with this, a recent prospective study in 200 neonates with DS showed that all of them had haematological abnormalities (Table 3) compared to neonates of the same gestational and post-natal age without DS

(Roberts et al, 2013). Furthermore, many of these abnormalities mirrored those seen in DS fetal liver haematopoiesis (see above) and were present even in the absence of mutations in *GATA1*. Neonates with DS had higher haemoglobin concentrations, increased circulating erythroblasts and abnormal red cell morphology, including macrocytosis, target cells and basophilic stippling; median platelet counts were also lower than normal in DS, thrombocytopenia was common and although the median MPV was similar to neonates without DS, platelet morphology was abnormal (giant platelets, circulating megakaryocytes and/or megakaryocyte fragments) in >95% of cases (Roberts et al, 2013). Interestingly, neonates with DS had higher numbers of granulocytes and monocytes despite the reduction in GMP in fetal liver (Roy et al, 2012) perhaps reflecting greater reliance on bone marrow haematopoiesis in late gestation and after birth. The total lymphocyte count was reduced in the DS neonates consistent with previous studies in older children with DS (de Hingh et al., 2005; Douglas, 2005; Verstegen et al, 2010).

Haematological abnormalities in older children and adults with DS: In contrast to the well-defined epidemiology of acute leukaemias, which in total affect ~3% of children with DS, little is known about the haematology of the majority of individuals with DS who do not develop leukaemia. The most commonly reported abnormalities are red cell macrocytosis (David*, et al* 1996, Roizen and Amarose 1993) and qualitative and quantitative abnormalities of B- and T-lymphocytes (Lin et al, 2001; de Hingh et al., 2005; Douglas, 2005; Garrison et al., 2005; Gillespie et al., 2006; Vertstegen et al, 2010).

The only large study, in 147 adults with DS (mean age 42.2 years; range 16-76 years), reported that these individuals had a higher mean MCV (99.28fL) compared to healthy controls and that almost 50% had a MCV above the upper limit of the normal range (Prasher, 1994). These data, with the consistent finding of macrocytosis in all of the mouse models of DS (Kirsammer et al, 2008; Carmichael et al, 2009; Alford et al, 2010), provide strong evidence for a role of abnormal expression of one or more genes on chromosome 21 (Hsa21) in the pathogenesis of the macrocytosis. Interestingly, of the 9 patients with DS investigated by McLean et al, two had a diagnosis of myelodysplasia, one of whom developed progressive bone marrow failure (McLean*, et al* 2009). The study by Prasher (1994) also commented that 7/147 (4%) individuals with DS had unexplained thrombocytopenia and 29/147 (20%) had unexplained neutropenia, none of whom had undergone haematological assessment, raising the possibility that a far greater proportion of adults with DS may have undiagnosed myelodysplasia or bone marrow failure than hitherto appreciated.

A number of studies have shown that children with DS have a high frequency of lymphopenia with a progressive decline in B and T cell lymphocyte numbers during childhood (de Hingh*, et al* 2005, Douglas 2005). Consistent with this, children with DS have increased susceptibility to infection and to autoimmune disorders (Garrison*, et al* 2005, Gillespie*, et al* 2006, Karlsson*, et al* 1998). B and T lymphocyte function may also be abnormal since small studies report evidence of abnormal thymic maturation, impaired lymphocyte activation and variable degrees of immunoglobulin deficiency (Lin*, et al* 2001, Loh*, et al* 1990, Murphy and Epstein 1990).

In summary, the occurrence of multilineage haematological abnormalities in neonates, children and adults with DS strongly suggest that cT21 perturbs haematopoiesis throughout life. The effects of cT21 differ at different stages of development. This may reflect age-related changes in the haematopoietic microenvironment, in the HSPC themselves or both.

Molecular basis for perturbation of haematopoiesis by cT21

The direct aetiological link between cT21 and leukaemia, both in DS and in leukaemias where T21 is an acquired abnormality (Izraeli 2005), provide a strong rationale for investigating how an additional copy of Hsa21 specifically alters HSPC development. T21 may exert its effects in several different ways. First, trisomic genes on Hsa21 may directly influence HSPC behaviour through gene dosage of one or more Hsa21 genes important for HSPC proliferation, differentiation or survival (Table 4) although a recent study makes it clear that Hsa21 potentially alters the expression of multiple genes on virtually any (or all) of the disomic chromosomes (Letourneau et al, 2014). These effects of Hsa21 may be haematopoietic cell autonomous and/or they may be mediated via other cell types, for example of the microenvironment. Secondly, the physical presence of an additional chromosome (as a cellular response to aneuploidy that may or may not be a specific response to T21) may alter the chromatin environment in some way thereby perturbing gene expression. For example widespread gene methylation changes were reported in ML-DS (Malinge*, et al* 2013). A recent study of mouse models and human DS-ALL revealed dramatic reduction of the repressive methylation of Histone 3 lysine 27, suggested to be

at least partially related to the increased expression of the Hsa21 gene HMGN1 (Lane*, et al* 2014). Furthermore the widespread gene expression abnormalities in disomic chromosomes in cT21 cells were shown to be arranged in specific domains suggesting an epigenetic chromatin regulatory mechanism (Letourneau*, et al* 2014). This phenomenon coupled with inter-individual differences in gene expression may mask those due to Hsa21 complicate the challenge of identifying the molecular basis of the effects of trisomy 21 on fetal haematopoiesis and leukaemia susceptibility.

In principle, an attractive approach to investigating leukaemia susceptibility is to study rare patients with partial (segmental) trisomy of Hsa21. Korbel et al used high resolution breakpoint mapping and oligonucleotide DNA tiling arrays in 30 individuals with DS due to segmental cT21 to try to identify discrete regions of Hsa21 associated with specific DS phenotypes (Korbel et al, 2009). They identified an 8.5Mb region on Hsa21 linked to leukaemia risk which included several genes, such as *RUNX1*, *ERG* and *ETS2*, known to be associated with acute leukaemia (Korbel et al, 2009). However, conclusions from this are limited so far by the very small number of cases of leukaemia in the study. A similar approach has been used extensively in a series of elegant transgenic mouse models containing additional copies of all, or part, of mouse chromosomal regions syntenic with Hsa21 (Kirsammer et al, 2008; Carmichael et al, 2009; Malinge et al, 2012; (Lane*, et al* 2014) or of human Hsa21 (Alford et al, 2010).

Using mouse models to study the impact of trisomy 21: Ts65Dn mice, which are trisomic for ~104 genes on mouse chromosome 16 (syntenic with Hsa21),

develop a myeloproliferative disorder linked to overexpression of *ERG* (Ng et al, 2010), although this manifests in adult rather than fetal or neonatal mice (Kirsammer et al, 2008). More recently, a double trans-genic mouse model was used to show that overexpression of *ERG* caused MEP expansion (analagous to that seen in human DS fetal liver) and that this synergised in vivo with expression of GATA1s to cause a TAM-like syndrome which then progressed to myeloid leukaemia (Birger et al, 2013). *ERG* has also been shown to promote megakaryopoiesis and induce megakaryoblastic leukaemia in the absence of a T21 background (Salek-Ardakani et al, 2009) although it is not yet clear that *ERG* is overexpressed in human cT21 fetal liver cells (Roy et al, 2012), hESC/iPSC (MacLean et al, 2012; Chou et al, 2012) or in ML-DS (Bourquin et al, 2006). *DYRK1A* has also been implicated in the pathogenesis of ML-DS. Using a refined mouse model (Ts1Rhr) trisomic for 33 Hsa21 orthologues, Malinge et al recently produced T21-dependent ML-DS by crossing Ts1Rhr mice with GATA1s knock in mice and over-expressing a transforming *MPL* allele (MPL^{W515L}) (Malinge et al, 2012). Using this model, both shRNA and gene expression profiling and functional studies identified *DYRK1A* (and possibly *CHAF1B*, *HLCS* and *ERG*) as mediators of abnormal megakaryopoiesis and showed that *DYRK1A* functioned as a megakaryoblastic tumour-promoting gene in the setting of partial T21 and *GATA1*s (Malinge et al, 2012). Using a similar approach Lane et al have recently identified Hmgn1 as a lymphoid leukaemia susceptibility gene in the Ts1Rhr mouse (Lane*, et al* 2014).

The Tc1 mouse model, the only mouse model which contains Human chromosome 21 genes, develops macrocytic anaemia, splenomegaly and

increased megakaryopoiesis but neither leukaemia nor a true myeloproliferative disorder (Alford et al, 2010). More recently, multiple structural rearrangements/deletions have been identified in Tc1 mice which may help to better refine the contribution of individual genes since it is now clear that 50 of the Hsa21 genes in this model, including *RUNX1*, are disomic (Gribble et al, 2013). Further studies using the newly characterised Tc1 mice (which have a copy of ~80% of Hsa21 genes), and other mouse models, are likely to provide important insight into the function of a number of Hsa 21 genes although none of these models yet fully recapitulates the human disease.

Patterns of gene expression in human trisomy 21 cells: Hsa21 has ~240 protein-coding genes and almost 340 short and long non-coding genes (www.ensembl.org/Homo_sapiens/Location/Chromosome?r=21). The functional correlation between levels of expression of Hsa21 genes and phenotype is not yet known and, as mentioned above, to a large extent will be tissue-specific and modulated by a number of factors, including cell lineage, developmental stage, differentiation status, cell cycle status, metabolic status and inter-individual differences.

For several cell types, including primary fetal heart and adult brain tissue and various fibroblast and lymphoblastoid cell lines as well as DS leukemias, an average 1.5-fold increased level of expression of Hsa21 genes in trisomic compared to disomic samples has been reported (Conti et al, 2007; Dauphinot et al, 2005; Prandini et al, 2007; Ait-Yahya-Graison et al, 2007; Lockstone et al, 2007; (Hertzberg*, et al* 2007, Hertzberg*, et al* 2010). However, it is already clear that the predicted 1.5 fold increase in expression of each individual Hsa21 gene

does not occur in any of the cell types investigated to date even if the average expression of all genes on Hsa21 is ~1.5-fold higher than disomic control cells (Prandoni et al, 2007). For example the tricistron that includes miR-125b-2 miR-99a and miR let7-c is expressed in ML-DS and is not expressed in DS-ALL while it is expressed in other cytogenetic subtypes of ALL disomic for Hsa21 (Emmrich*, et al* 2014, Gefen*, et al* 2010). One of the strongest pieces of evidence linking the level of expression of Hsa21 genes to cellular phenotype comes from very elegant experiments in which introduction of an inducible Xist transgene into the *DYRK1A* locus on Hsa21 in DS iPSC successfully induced transcriptional silencing of the genes on that chromosome in tandem with reversal of the trisomy 21-associated in vitro defects of neural cell development (Jiang et al, 2014). The effects on haematopoietic differentiation were not reported in this study and might not have been so dramatic given that differentiated trisomic human iPSC appear to display subtle, or no, differences in Hsa21 gene expression compared to their disomic counterparts despite marked differences in their haematopoietic phenotype (MacLean et al, 2012; Chou et al, 2012).

Nevertheless, Hsa21 contains a relatively large number of Hsa21 genes which are either implicated in haematological malignancies (eg *CSTB*, *DYRK1A*, *ERG*, *ETS2*, *OLIG2*, *RUNX1* and *TIAM1*) or encode proteins known to play an important role in haematopoiesis (*AIRE*, *BACH1*, *CBG*, *DNMT3L*, *GABPA*, *IFNAR1*, *IFNAR2*, *IFNGR2*, *RCAN1*, *SOD1* and *SON*) (Elagib et al, 2003; Toki et al, 2005; Xu et al, 2006; Yu et al, 2011; Malinge et al, 2012; Birger et al, 2013). There are also 5 microRNAs (miRs) on Hsa21, four of which are expressed in megakaryocyte lineage cells (Garzon et al, 2006; Klusmann et al,

2010b; Emmrich*, et al* 2014). *RUNX1*, for example, is a tumour suppressor in AML in individuals without DS (Schnittger et al, 2011) and the three copies in cT21 are inconsistent with development of leukaemia. Indeed *RUNX1* expression is *lower* in ML-DS compared with non-DS AMKL (Bourquin et al, 2006), and is not increased in in fetal liver CD34+ cells (Roy et al, 2012) or cT21-derived hESC or iPSC (MacLean et al, 2012; Chou et al, 2012). Similarly, *ERG*, a potent megakaryoblastic oncogene in murine models, as discussed above, has not yet been found to be increased in human cT21 fetal haematopoietic or leukaemic cells (Roy et al, 2012; Bourquin et al, 2006). In summary, although there is no direct evidence that cT21-associated changes in expression of any of these genes in primary haematopoietic cells is able to induce leukaemic transformation, they remain important candidate genes to investigate as their role may only be evident by looking in well-defined HSC or progenitor populations.

Summary and conclusions

Recent data from primary human fetal liver, as well as hESC and iPSC, show that T21 itself alters human fetal HSC and progenitor biology causing multiple defects in megakaryocyte/erythroid and B lymphoid lineage development. These data provide clues to mechanisms by which T21, or aneuploidy in general, may perturb haematopoietic cell growth and differentiation and a model with which to investigate these. The molecular basis of these effects is likely to be complex, to be both tissue- and lineage-specific and to be dependent on the fetal liver, and possibly bone marrow, microenvironment.

How these abnormalities set the stage for haematological malignancies in DS is clearer for the myeloid leukaemias but less well understood for the lymphoid leukaemias (Table 5). There is good evidence that cT21 driven fetal expansion of mega-erythroid stem and progenitor cells predisposes to malignant transformation by an acquired mutated GATA1s protein. It is possible that the relative block in B cell development, together with the associated epigenetic abnormalities and enhanced sensitivity of these B cell precursors to malignant transformation, as suggested by mouse models, explains the high prevalence of lymphoid leukaemias in DS.

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Figure legends

Figure 1: Peripheral blood film from a neonate with Down syndrome and a clinical and haematological diagnosis of TAM confirmed by the presence of a mutation in exon 2 of the *GATA1* gene.

The film shows large numbers of blasts as well as giant platelets and circulating megakaryocytes.

Figure 2: Increased megakaryopoiesis in cT21 fetal liver in the absence of acquired *GATA1* mutations.

Representative image from a second trimester cT21 fetal liver paraffin-embedded section stained with the megakaryocyte marker CD42b (brown).

Table 1: Clinical features of TAM*

Clinical features	Comments
Jaundice	Very common. Although present in the majority of neonates with TAM, jaundice is also seen in almost 50% of DS neonates without TAM.
Hepatosplenomegaly	Common. Hepatomegaly or hepatosplenomegaly is more common than isolated splenomegaly
Rash	Uncommon. Non-specific appearance. Presence of a rash is highly suggestive of TAM although rashes also occur in DS neonates without TAM.
Pleural and/or pericardial effusions, and/or ascites	Effusions are reported in up to 25% of cases of clinically overt TAM.
Bleeding diathesis	Bleeding occurs in ~10% of cases; the cause is multifactorial and includes thrombocytopenia, liver dysfunction and disseminated intravascular coagulation.
Hepatic fibrosis with or without hepatic failure	Uncommon. Occasional neonates present with hepatic fibrosis despite relatively low numbers of circulating peripheral blood blasts.
Renal dysfunction/ renal failure	Uncommon (less than 10%)
Hydrops fetalis	Although TAM is rarely reported to present as hydrops fetalis, some cases of TAM may be missed as *GATA1* mutation analysis is not always performed.
Asymptomatic	~20% of neonates with TAM have no typical clinical features of TAM

* Clinical features seen in neonates with Down syndrome who have a *GATA1* mutation detectable by direct sequencing and/or direct high performance liquid chromatography (dHPLC). These data have been compiled using data from Klusmann et al, 2008; Gamis et al, 2011; and Roberts et al, 2013.

Table 2: Haematological features of TAM*

Haematological features	**Comments**
Leucocytosis	Common. Although present in the majority of neonates with TAM, leucocytosis is also seen in many DS neonates without TAM. Similarly the leucocyte count is normal in at least a third of neonates with TAM
Peripheral blood blasts >20%	Very common. Since ~all neonates with DS have peripheral blood blasts and ~20% of neonates with TAM have <20% blasts, the significance of the presence of blast cells on the peripheral blood film can only be determined by carrying out *GATA1* mutation analysis (see text).
Neutrophilia	Common. Neutrophilia is also found in 25% of neonates with DS who do not have a *GATA1* mutation.
Eosinophilia	Uncommon (10-16%)
Thrombocytopenia	Common. Thrombocytopenia occurs at a similar frequency in TAM as in DS neonates without a *GATA1* mutation and more than one third of neonates with TAM are not thrombocytopenic
Anaemia	Uncommon (~10%)

* Haematological features seen in neonates with Down syndrome who have a *GATA1* mutation detectable by direct sequencing and/or direct high performance liquid chromatography. These data have been compiled using data from Klusmann et al, 2008; Gamis et al, 2011; Roberts et al, 2013; and Maroz et al, 2014.

Table 3: Haematological abnormalities in neonates with Down syndrome compared to neonates without Down syndrome

Haematological abnormality
Erythropoiesis - increased haemoglobin and haematocrit - increased MCV - peripheral blood erythroblastosis - dyserythropoiesis (eg target cells, macrocytes, basophilic stippling)
Leucocytes - increased leucocytes - increased neutrophils - increased monocytes - increased basophils - increased peripheral blood blasts - reduced lymphocytes - dysplastic neutrophils and monocytes
Platelets - reduced platelet count - giant platelets - circulating megakaryocytes and/or megakaryocyte fragments

Table 4: Genes on human chromosome 21 with known functions in haematopoietic cells

Genes implicated in haematological malignancies
CSTB *DYRK1A* *ERG* *ETS2* *OLIG2* *RUNX1* *TIAM*
Other genes relevant to haematopoiesis
AIRE *BACH1* *CBG* *DNMT3L* *GABPA* *IFNAR1, IFNR2* and *IFNG2* *RCAN1* *SOD1* *SON*

Table 5: Proposed pre-leukemic hematopoietic defects caused by cT21 in Down Syndrome*

Myeloid Compartment	• Fetal liver expansion of hematopoietic stem cells and megakaryocytic-erythroid progenitors • DNA promoter hypomethylation • Increased expression of genes implicated in eryhto-megakaryocytic development and transformation • Suppression of the NFAT pathway by DYRK1a and RCAN1 overexpression
Lymphoid Compartment	• Fetal B cell developmental defect with accumulation of pro-B progenitors • Increased transformability of cT21 B cell progenitors • Increased V(D)J mediated chromosomal rearrangements (e.g CRLF2) due to developmental arrest in precursor B cell stage. • Decreased tri-methylation of lysine 27 of histone 3, possibly caused by Increased expression of HMGN1 • Suppression of NFAT pathway may explain the rarity of T-ALLs.

* Based on somewhat speculative interpretation of studies of mouse and human models described in this review.