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This work was supported by a research grant from Novo Nordisk.

Disclosure Summary: The authors have nothing to declare.

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Review

doi: 10.4168/aair.2011.3.2.81
pISSN 2092-7355 • eISSN 2092-7363

Allergy Asthma & Immunology Research

IL-33 and Airway Inflammation

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Interleukin-33 (IL-33) is the 11th member of IL-1 cytokine family which includes IL-1 and IL-18. Unlike IL-1β and IL-18, IL-33 is suggested to function as an alarmin that is released upon endothelial or epithelial cell damage and may not enhance acquired immune responses through activation of inflammasome. ST2, a IL-33 receptor component, is preferentially expressed by T-helper type (Th) 2 cells, mast cells, eosinophils and basophils, compared to Th1 cells, Th17 cells and neutrophils. Thus, IL-33 profoundly enhances allergic inflammation through increased expression of proallergic cytokines and chemokines. Indeed, IL-33 and its receptor genes are recognized as the most susceptible genes for asthma by several recent genome-wide association studies. It has also recently been shown that IL-33 plays a crucial role in innate eosinophilic airway inflammation rather than acquired immune responses such as IgE production. As such, IL-33 provides a unique therapeutic way for asthma, i.e., ameliorating innate airway inflammation.

Key Words: IL-33; ST2; host defense; allergy; autoimmunity; chronic disease, mast cell; basophil; eosinophil

Identification of a nuclear protein and an orphan receptor as IL-33 and IL-33 receptor

Interleukin-33 (IL-33), a member of the IL-1 cytokine family, is considered to be crucial for the induction of T-helper type (Th) 2 cell-dominant immune responses such as host defense against nematodes and allergic diseases.[1] IL-33 was originally identified as "DVS27", a gene upregulated in vasospastic cerebral arteries after subarachnoid hemorrhage[2] and as a nuclear factor, "nuclear factor from high endothelial venules (NF-HEV)", which is expressed in endothelial cell nuclei.[3]

IL-33 receptor was first identified as an IL-1 receptor-like molecule and termed as ST2 (the gene symbol was termed as *IL1RL1*) by Tominaga in 1989.[4] ST2 was subsequently found to be preferentially expressed in Th2 cells and started to attract many researchers involved in allergy.[5] In 2005, DVS27 was rediscovered as the 11th member in the IL-1 family of cytokines, which includes IL-1α, IL-1β, and IL-18, by computationally searching for the sequences containing β-trefoil structure seen in IL-1- and FGF-like proteins, and termed as IL1F11 or IL-33.[6]

IL-33 receptor and signal transduction

As the receptors for the other IL-1-related cytokines, the IL-33 receptor is formed heterodimeric molecules consisting of ST2 and IL-1 receptor accessory protein (IL-1RAcP; Fig. 1). IL-1RAcP is also known as a common component of receptors for IL-1α, IL-1β, IL-1F6, IL-1F8, and IL-1F9.[1]

The two major products of ST2 genes (*IL1RL1*), i.e., transmembrane form ST2 (ST2 or ST2L) and soluble form ST2 (sST2) are produced by alternative splicing under the control of two distinct promoters. ST2 is considered to be the functional component for induction of IL-33 bioactivities, while sST2 act as a decoy receptor for IL-33 like soluble IL-1Rs for IL-1.[3]

The signal transduction downstream of IL-33 receptor is mediated by common adapter molecules to that of the other IL-1 receptor family such as IL-1R and IL-18R. The binding of IL-33 to IL-33 receptor results in the recruitment of MyD88 to the Toll-interleukin-1 receptor domain in cytoplasmic region of ST2, leading to the induction of inflammatory mediators by activating transcription factors such as NF-κB and AP-1 through IRAK, TRAF6 and/or MAP kinases, like other IL-1 family receptor or Toll-like receptor (TLR) activation.[6]

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Received: December 31, 2010; Accepted: January 4, 2011
There are no financial or other issues that might lead to conflict of interest.

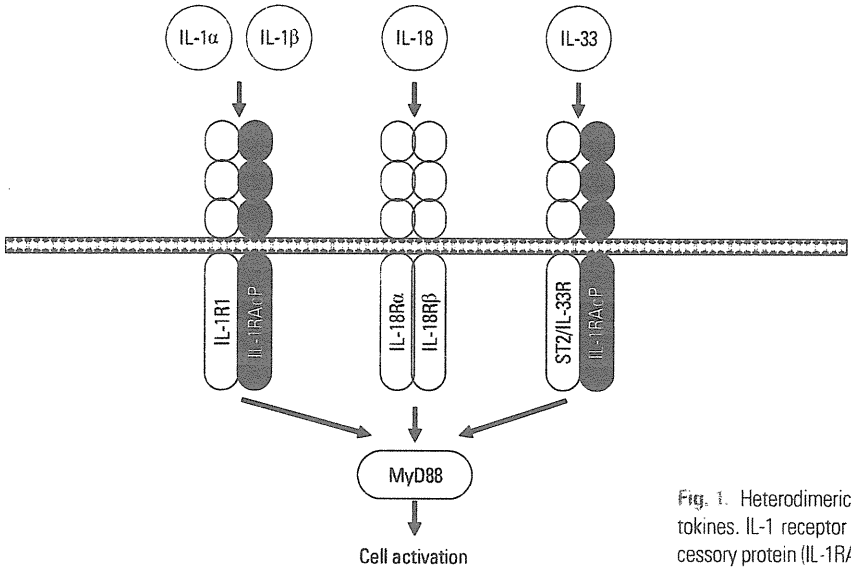

Fig. 1. Heterodimeric receptors for interleukin-1 (IL-1) family cytokines. IL-1 receptor and IL-33 receptor share IL-1 receptor accessory protein (IL-1RAcP) amplifying the receptor signaling.

THE SOURCES AND THE TARGETS OF IL-33

IL-33 as a alarmin

In contrast to the other IL-1 family cytokines except IL-1α, IL-33 is localized to the nucleus of human epithelial and endothelial cells[2] and mouse bone-marrow derived cultured mast cells[7] by binding to chromatin via a homeodomain (helix-turn-helix-like motif) and nuclear localization signal in amino-terminus.[8] Although the pathophysiological role of IL-33 as a nuclear factor is not fully understood, IL-33 binds to the acidic pocket of dimeric histone H2A-H2B at the surface of nucleosomes, resulting in the suppression of the gene transcription at least in the *in vitro* reporter assay system.[8]

During host defense against pathogens, innate-type immune cells recognize pathogen-associated molecular patterns via TLRs, resulting in induction of inflammation. In addition, endogenous proinflammatory factors called "damage associated molecular patterns (DAMPs)" (also called "alarmin"), which are released by necrotic cells in tissue injury during trauma and/or infection, also provoke local and/or systemic inflammation by alerting acquired-type immune cells as an endogenous danger signal.[9] For example, high-mobility group box 1 (HMGB1), which is originally identified as a nuclear factor as a transcriptional regulator, is released by macrophages in response to lipopolysaccharide, leading to the induction of inflammation.[10] Like HMGB1, recent several lines of evidence suggest that IL-33, which also localizes in nucleus, act as a DAMP/alarmin.[11]

IL-33 was originally considered to be secreted by the activation of NACHT, LRR and PYD containing protein (NLRP)-mediated inflammasomes like IL-1β and IL-18 since it is cleaved from pro-IL-33 by caspase-1 *in vitro*.[6] However, pro-IL-33 does not have a typical cleavage site seen in pro-IL-1β and -IL-18, and caspase-1 was found to proteolytically cleave pro-IL-33 at the cytokine motif, but not the intermediate region between helix-turn-helix motif and cytokine motif, resulting in the inactivation of IL-33.[11-13] Like caspase-1, both caspase-3 and caspase-7 have an ability to cleave pro-IL-33 during apoptosis, in which apoptotic cells do not induce inflammation generally, and the processed IL-33 by these caspases do not have biological activities.[11,12]

On the other hand, biologically active pro-IL-33 can be released by necrotic cells without any processes by caspase-1, -3, -7, -8, and calpain.[7,11-13] For example, pro-IL-33 can induce mouse mast cell activation to produce cytokines.[13] These observations suggest that pro-IL-33 released by necrotic cells during tissue injury may have a potential role in induction of inflammation as a DAMP/alarmin.

IL-33 is an epithelial-mesenchymal cytokine manipulating inflammatory responses

Asthma is an inflammatory disease characterized by infiltration of the airway wall with a variety of immune cells and inflammatory cells such as Th2 cells, mast cells and eosinophils. However, a key component of asthma is the structural change that involves all of the elements of the airway wall associated with activation of the epithelial-mesenchymal trophic unit.[14]

Activated epithelial and mesenchymal cells generate a range of growth factors associated with airway remodeling and cytokines manipulating the immune response. Although IL-33 is present in the nuclei of various cell types, epithelial cells[15] and endothelial cells[16] are recognized as the major sources of the cytokine especially when considering the event of tissue damage.

Thus, like thymic stromal lymphopoietin (TSLP),[17] IL-33 is

now recognized as an epithelial-mesenchymal-derived cytokine manipulating inflammatory and/or immune responses.

Target cells of IL-33

Th2 cells

It is well established that IL-4 is a key cytokine for the differentiation of Th2 cells from naïve $CD4^+$ T cells. ST2 is predominantly expressed on Th2 cells but not naïve T cells, Th1 cells, Th17 cells and regulatory T cells.[18-20] On the other hand, ST2 is not essential for Th2 cell differentiation as shown in the study using ST2-deficient mice; ST2-deficient mice showed the normal development of Th2 cells.[21,22] In support for the notion, although IL-33 cannot induce the differentiation of Th2 cells from naïve $CD4^+$ T cells *in vitro*,[23,24] IL-33 can enhance IL-5 and IL-13 production by *in vitro*-skewed Th2 cells which highly express ST2.[6,25,26] Also, Kurowska-Stolarska et al.[24] reported that IL-33 induces the differentiation of $IL\text{-}5^+IL\text{-}4^-$ $CD4^+$ Th cells from naïve $CD4^+$ T cells independently of IL-4, STAT-6 and GATA-3, which are important factors for the typical Th2 cell differentiation.

In human, IL-33 potentiates not only Th2-type cytokine production but also Th1-type cytokine, IFN-γ, production by peripheral blood-derived Th2 cells.[27] In addition, IL-33 acts as a chemoattractant for Th2 cells, but not Th1 cells, in both human and mice.[28]

Innate lymphoid cells

Lin- $c\text{-}Kit^+$ $Sca\text{-}1^+$ natural helper cells dwelling in the gut adipose tissue are a newly identified.[29] Natural helper cells constitutively express ST2 and can produce a larger amount of IL-5 and IL-13 rather than basophils and mast cells in response to IL-33. It was shown that IL-33-mediated natural helper cell activation was important for formation of goblet cell hyperplasia during *Nippostrongylus brasiliensis* infection.[29] Similar ST2-expressing non T/non B lymphoid cell types capable of producing IL-5 and IL-13 in response to IL-33, are subsequently identified by other investigators.[30,31]

Mast cells

Mast cells, which dwell in the mucosal and connective tissues, express c-Kit and high affinity IgE receptors (FcεRI), and induce IgE-mediated immune responses, are also major targets of IL-33. Mouse and human mast cells constitutively express ST2.[32-35] Except IL-3 and stem cell factor (SCF, a ligand for c-kit), which are required for mast cell development at least in mouse, IL-33 is the only cytokine among 45 different cytokines which can directly provoke cytokine/chemokine (IL-1β, IL-6, IL-13, TNF, and MCP-1) secretion from mouse bone-marrow derived cultured mast cells without affecting their degranulation.[36,37]

Like a murine counterpart, IL-33 can induce cytokine and chemokine production, prolong survival and promote cell-adhesion in human cord blood stem cell-derived cultured mast cells.[34,35] In addition, IL-33 can augment IgE-mediated cytokine production and degranulation by mouse bone-marrow derived cultured mast cells and human cord blood stem cell-derived cultured mast cells.[34-36,38]

Basophils

Another highly FcεRI-expressing cell type, basophils, which circulate in the peripheral blood and are potential primary sources of IL-4,[39,40] are also considered as the major target of IL-33. In comparison with Th2 cells and mast cells, human and mouse basophils constitutively express ST2 at the relatively low level on their cell surface.[23,26,41,42] On the other hand, the expression of ST2 on the cell surface of basophils is promoted by stimulation with IL-3.[26]

Like the effect of IL-33 on Th2 cells and mast cells, IL-33 alone can induce the production of cytokine including Th2-type cytokines and chemokines by basophils and promote cell-adhesion and CD11b expression in basophils in human or mice.[26,27,41,42] IL-33 does not induce degranulation by basophils directly, while IL-33 synergistically enhances IgE-mediated degranulation by human basophils.[26,41]

In addition, IL-33 augments immune responses of basophils in human or mice; eotaxin-mediated migration,[41] cytokine secretion in the presence of IL-3, which is a growth factor for basophils like mast cells,[23,26,27,41-43] and prolongs survival in the presence of IL-3 or GM-CSF.[41-43] These observations suggest that IL-33 is a potential activator for basophils by enhancing cytokine and chemokine secretion, recruitment and adhesion.

Eosinophils

Peripheral blood eosinophils, compared to neutrophils, are preferentially recruited into the tissue at the site of inflammation in patients with certain IgE-mediated allergic disorders such as asthma. Although ST2 expression was barely detectable on cell surface of peripheral blood eosinophils in human, ST2 mRNA and intracellular ST2 protein were detectable in them.[26,44,45] IL-33 can induce the production of superoxide and IL-8 directly, and enhance IL-3, IL-5 or GM-CSF-mediated IL-8 production by human eosinophils.[26,44]

Like mast cells and basophils, IL-33 enhances adhesion of eosinophils by promoting CD11b expression and survival independently of IL-4, IL-5, and GM-CSF.[45] Unlike basophils, IL-33 did not influence eotaxin-mediated migration of eosinophils.[45] These observations strongly suggest that IL-33 may contribute to the pathogenesis of certain allergic disorders accompanied by marked accumulation of eosinophils.

Dendritic cells

IL-33 promotes the development of dendritic cells (DCs) from bone marrow cells.[46] It has been shown that DCs derived by the cultivation of murine bone marrow cells in the presence of GM-CSF and IL-4 (that is, bone marrow-derived DCs; BMDCs) express ST2.[47] IL-33 enhances the production of IL-6, but not IL-

12, by BMDCs and augments the expression of MHC class II and CD86, but not CD80, CD40 and OX40 ligand (OX40L), on the cell surface of BMDCs.[47]

When naive CD4+ T cells were co-cultured with BMDCs in the presence of IL-33 for 6 to 10 days, IL-5 and IL-13, but not IL-4 and IFN-γ, were detected in the culture supernatant even without TCR engagements. Since the secreting cytokine profiles (IL-5 and IL-13, but not IL-4, production) in the settings (BMDCs + naïve CD4+ T cells + IL-33, no antigens) are similar to those by IL-5-positive IL-4-negative atypical Th2 cell population or innate lymphoid cell types, IL-33 may enhance the induction of these cell types from naïve CD4+ T cells.

Like IL-33, IL-25 and TSLP are known to be epithelial/mesenchymal cytokines inducing Th2-type cytokine-mediated immune responses.[48] Contrast to IL-33, TSLP-activated DCs promotes IL-4-producing Th2 cell differentiation from naïve CD4+ T cells in the presence of TCR engagements through OX40L-OX40 interaction at least in part.[49,50] IL-25 can enhance TSLP-stimulated DC-mediated Th2 cell expansion.[51]

Unlike IL-33, both TSLP and IL-25 can induce the differentiation of IL-4-producing Th2 cells from naïve CD4+ T cells after TCR engagements dependently of IL-4-IL-4Rα-STAT6 pathway.[52,53] Therefore, these observations suggest that the role of IL-33, TSLP, and IL-25 in T cells and DCs may be different in Th2-type cytokine-mediated immune responses; TSLP and IL-25 may be involved in the preferential induction of antigen-specific IL-4/IL-5/IL-13-producing Th2 cell-mediated immune responses, while IL-33 may contribute to the induction of antigen-non specific Th2 cell-mediated immune responses by inducing IL-5/IL-13-, but not IL-4-, producing atypical Th2 cells or innate lymphoid cells.

Other cell types

Regarding epithelial mesenchymal cell types, Yagami et al.[54] have examined IL-33-responsive cells among primary human lung tissue cells. They found that ST2 mRNA was expressed in both endothelial and epithelial cells but not in fibroblasts or smooth muscle cells. Correspondingly, IL-33 promoted IL-8 production by both endothelial and epithelial cells but not by fibroblasts or smooth muscle cells. Transfection of ST2 small interference RNA into both endothelial and epithelial cells significantly reduced the IL-33-dependent upregulation of IL-8, suggesting that IL-33-mediated responses in these cells occur via the ST2 receptor.

While Th2 cytokines, such as IL-4, further enhanced ST2 expression and function in both endothelial and epithelial cells, Th2/eosinophil-related cytokines/chemokines were not produced by these cell types. While the IL-33-mediated production of IL-8 by epithelial cells was almost completely suppressed by corticosteroid treatment, the effect of corticosteroid treatment on the IL-33-mediated responses of endothelial cells was only partial.[54]

Comprehensive role of IL-33 regarding other cell types and diseases other than asthma has been shown in our previous review article.[1]

IL-33 AND ASTHMA

Lessons from GWAS for asthma

The completion of the Human Genome Project, the HapMap project, and technological advances[55] allowed genome-wide association studies (GWAS) to more comprehensively identify the susceptibility genes for asthma. Although asthma is now recognized as a syndrome consisting of heterogeneous disease entities,[56] several genes are shown to be susceptible for asthma in GWAS. Recent large-scale GWAS all show the genes for IL-33 (*IL33*) and ST2 (*IL1RL1*) are susceptible for asthma onset.[57-61] It should be noted that *IL33* and *IL1RL1* are located in different chromosomes, and that only these two genes are consistently listed as asthma-susceptible genes in these literatures. Interestingly, susceptible genes for atopy (IgE production) were entirely different from those for asthma including *IL33* and *IL1RL1*.[58]

Thus, IL-33 now attracts much attention from all doctors and investigators who are involved in asthma research.

Role of IL-33/ST2 in asthma

The levels of soluble ST2 proteins and IL-33 mRNA/proteins are increased in sera and tissues from patients with asthma.[27,62-65] Also, intraperitoneal or intranasal administration of IL-33 in mice leads to the induction of inflammation accompanied by eosinophils in mucosa of lung and intestine through the IL-13 and STAT6-dependent pathway.[6,23] The levels of soluble ST2 protein and IL-33 mRNA are increased in sera and/or lungs in a murine asthma model of airway inflammation induced by ovalbumin (OVA).[66,67]

However, the role of ST2 and IL-33 in the induction of OVA-induced airway inflammation in mice is controversial. Especially, apparent discrepancy is often found between the studies using ST2-deficient mice and the studies using mice treated with anti-ST2 and ST2-Fc fusion proteins.[1]

Respiratory function, eosinophilic airway inflammation and the levels of serum total IgG1 and IgE were normally observed in 129×B6 mixed and BALB/c background-ST2-deficient mice sensitized twice with OVA emulsified with alum.[21,24,68] Several investigators reported the effect of anti-ST2 mAb (clone 3E10) on OVA-induced airway inflammation in BALB/c mice (twice sensitization model with OVA/alum). Airway inflammation induced by OVA was attenuated in BALB/c mice treated with the 3E10 anti-ST2 mAb.[69,70] Likewise, Th2 responses during OVA-induced airway inflammation were reduced in mice treated with anti-IL-33 polyclonal Ab.[71]

Adoptive transfer with DO11.10 Th2 cells, which express OVA-specific T cell receptors, into mice results in Th2 cytokine-dependent eosinophilic airway inflammation after intranasal OVA

challenge.[72] The airway inflammation was exacerbated when BALB/c wild-type mice or BALB/c-Rag-1-deficient mice injected with ST2-deficient DO11.10 Th2 cells were challenged with OVA in comparison with those mice injected with ST2-sufficient DO11.10 Th2 cells.[68] It suggests that IL-33 signals on Th2 cells may have a regulatory function in OVA-induced airway inflammation. Contrast with the study using ST2-deficient DO11.10 Th2 cells,[68] administration of the 3E10 anti-ST2 mAb or soluble ST2-Fc fusion protein in mice injected with DO11.10 Th2 cells showed the attenuated airway function and inflammation after OVA challenge.[19,70]

The reason for this discrepancy still remains unclear. However, it will be clarified by examining as to whether the expression of IL1RAcP, which is a signal amplifier and forms receptor complex not only with ST2 but also with IL-1 receptor (IL1R), is excessive in various immune cell types from ST2-deficient mice (Fig. 1).

Lessons from IL-33 gene deficient mice

Since the possibility of excessive IL-1 signaling in ST2 deficient mice cannot be ruled out, generation of IL-33 deficient mice has long been expected. Oboki et al.[73] have only recently generated the IL-33 deficient mice. These mice normally develop, suggesting that IL-33 does not play a crucial role as a nuclear factor in physiological development.

During airway inflammation induced by twice OVA with alum sensitization, IL-33$^{-/-}$ mice showed attenuated eosinophil influx into the bronchoalveolar lavage (BAL) fluid and pulmonary inflammation. In contrast, IL-4 and IL-5 levels in the BAL fluid and serum OVA-specific IgE production were only slightly (i.e., not significantly) reduced in IL-33$^{-/-}$ mice after the last OVA challenge. IL-33-deficiency also significantly diminished inflammatory cell influx into the BAL fluid during airway inflammation induced by an extract derived house dust mites (HDM).[73]

Furthermore, inhalation of papain, a cystein protease allergen having strong homology with HDM allergen, Der 1,[74] which can induce airway inflammation even in T/B cell-deficient Rag-2$^{-/-}$ mice, induced strong airway eosinophilia even without sensitization process. Most importantly, papain-induced airway inflammation is profoundly abolished in IL-33$^{-/-}$ mice as well as IL-4$^{-/-}$IL-13$^{-/-}$ mice. Therefore, the papain-induced innate airway inflammation is IL-33-dependent, and that IL-4 and/or IL-13 derived from innate inflammatory cells but not T cells are important for the event.[73]

Taken together, IL-33 is important for inducing antigen-dependent Th2-associated local airway inflammation. However, unlike TSLP[17] and IL-1, IL-33 is mostly dispensable for antigen-specific Th2 cell differentiation and antigen-specific IgE production (Fig. 2), although the actual asthma pathogenesis cannot be compartmentalized; i.e., IL-33 is capable of stimulating TSLP production.[75]

Oboki et al.[73] also found that IL-33 is involved in the development of dextran-induced colitis accompanied by T cell-independent epithelial cell damage, but not in streptozocin-induced diabetes or Con A-induced hepatitis characterized by T cell-mediated apoptotic tissue destruction. In addition, IL-33 failed

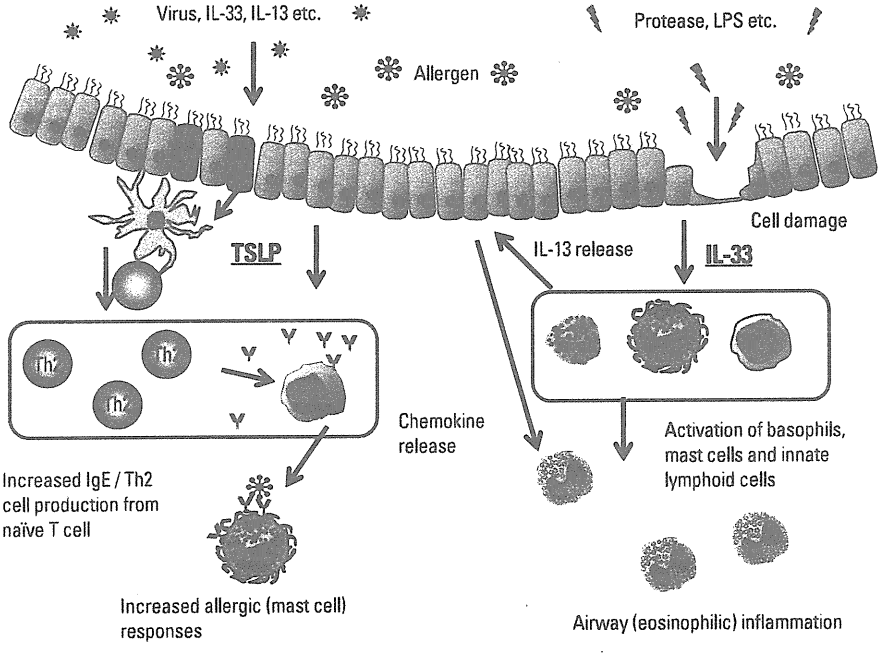

Fig. 2. The major role of interleukin-33 (IL-33) in asthma pathogenesis in comparison with thymic stromal lymphopoietin (TSLP). IL-33 is crucial for protease-mediated innate airway inflammation associated with cell damage, while TSLP profoundly affect the allergen-specific IgE production/Th2 differentiation.

to play a substantial role in induction of T cell mediated contact and delayed-type hypersensitivity and autoimmune diseases. Of note, unnatural up-regulation of acquired immunity often seen in ST2-deficient mice is not seen in IL-33-deficient mice.[73]

CONCLUSION

Since recent large-scale GWAS all show the genes for IL-33 and its receptor are susceptible for asthma, IL-33 now attracts much attention from all investigators involved in asthma research. IL-33 is important for protease-mediated innate airway inflammation and in the late-phase inflammatory responses in the lung observed after IgE-mediated reaction without affecting acquisition of antigen-specific memory T cells. Thus, IL-33 provides a unique therapeutic way for asthma, i.e., ameliorating innate airway inflammation.

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European Journal of Cancer (2012) xxx, xxx–xxx

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Is the prognosis of stage 4s neuroblastoma in patients 12 months of age and older really excellent?

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KEYWORDS
Neuroblastoma
Stage 4s
Stage MS
Prognosis

Abstract *Purpose:* In the International Neuroblastoma Risk Group (INRG) classification system, stage 4s was changed into stage MS in children less than 18 months of age. Stage MS is defined as a metastatic disease with skin, liver and bone marrow, similar to INSS stage 4s. To evaluate the outcome of stage 4s cases in patients 12 months of age and over and to determine the appropriate treatment strategy.

Method: We performed a retrospective review of 3834 patients registered with the Japanese Society of Pediatric Oncology and Japanese Society of Pediatric Surgeons between 1980 and 1998.

Results: The rates of stage 4s patients were 10.7%, 6.3% and 3.3% in patients of $\leqslant$11 months of age, from $\geqslant$12 to $\leqslant$17 months of age, $\geqslant$18 months of age, respectively. The 5 year event-free survival rates were 89.4%, 100% and 53.1%, respectively. The rates of *MYCN* amplification and unfavourable histology were smaller in stage 4s groups than stage 4 groups in all ages.

Conclusion: In the children 12 months of age and older, stage 4s cases are markedly different from stage 4 cases in regard to the clinical features and prognosis. The prognosis of stage 4s cases from $\geqslant$12 to $\leqslant$17 months of age is excellent. The concept of stage MS appears to be appropriate.

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doi:10.1016/j.ejca.2012.01.010

Please cite this article in press as: Iehara T. et al., Is the prognosis of stage 4s neuroblastoma in patients 12 months of age and older really excellent? *Eur J Cancer* (2012), doi:10.1016/j.ejca.2012.01.010

1. Introduction

Neuroblastoma is the most common extra cranial solid tumour in childhood. The prognosis of the disease is largely dependent on the age of the child and the extension of the tumour at diagnosis. In general, the prognosis for neuroblastoma in infants is much more favourable than in older children. Stage 4s neuroblastoma, first described by Evans, is a special metastatic disease for patients <12-months-old associated with a favourable prognosis.[1,2] Although these patients have wide metastatic disease, they have a favourable prognosis and also have high rates of a spontaneous regression. The stage 4s neuroblastoma is defined as an infant <12-months-old with metastases restricted to the liver, skin, and/or bone marrow, in which the primary tumour is localised (stage 1 or 2).

Recently, the International Neuroblastoma Risk Group (INRG) classification system was developed in order to establish a consensus approach for pre-treatment risk stratification. The new International Neuroblastoma Risk Group Staging System (INRGSS) was developed for the INRG.[3,4] To classify neuroblastoma patients by INRG classification system, we used the criteria of INRG stage, age, histological category, grade of tumour differentiation, *MYCN* status, 11q aberrations and tumour cell ploidy. In this INRG system, stage 4s changes to stage MS in children <18 months old. Stage MS is defined as a metastatic disease with special features, similar to INSS stage 4s, although there is no restriction regarding the size of the primary tumour. The metastases are restricted to the skin, liver and bone marrow. Age is not a component of the definition of stage MS.[3,4] Therefore, stage MS includes children aged from more than 12 months to less than 18 months of age. Conventionally, an age of ⩾12 months has been the reference point for decisions for stage 4 neuroblastoma. Recently, an age cutoff of 18 months was proposed in a large-scale research study.[5]

The present study was undertaken to clarify how high the frequency of the stage 4s cases of ⩾12 months and to clarify whether the prognosis is excellent or not for a decision-appropriate treatment strategy. This is the first report of stage 4s neuroblastoma in patients ⩾12 months of age.

2. Patients and method

A retrospective review of 3834 patients with neuroblastoma was performed. The patients were registered with the Committee of Neuroblastoma in the Japanese Society of Pediatric Oncology and Japanese Society of Pediatric Surgeons between 1980 and 1998.

The patients were divided into three groups: ⩽11-months of age, ⩾12 to ⩽17-months of age and ⩾18-months of age.

From these three groups, we extracted the cases suited for stage 4s. The cases of metastasis were limited to the skin, liver or bone marrow. The primary tumours were observed within the tumour capsule (C_1) or outside the tumour capsule but not beyond the midline (C_2) and without contra lateral regional lymph node, in other words, the tumour of stage 3 was omitted. The maximum diameter of the primary tumours is less than 10 cm. In infancy, the stage 4s definition excluded bone marrow metastasis with more than 10% tumour cell infiltration. However, in this study, the ratio of the infiltration tumour cells is not considered in the stage 4s cases >12 months old. Thereafter, we examined the frequency of these extracted cases and compared the clinical feature and prognosis of stage 4 with those of 4s cases.

The stage 4s cases <12 months old were given either six cycles of the low-dose regimen, consisted of a low-dose of cyclophosphamide and vincristine over a 2-week period to shrink the tumour, followed by surgical resection. Stage 4 cases were treated with intensive chemotherapy consisting of cyclophosphamide and pirarubicin, cisplatin, vincristine or etoposide. Infants less than 12-months old were treated with reduced dosages. After 1992, many cases, especially cases with *MYCN* amplification, received high-dose chemotherapy with stem cell transplantation.

Amplification of the *MYCN* had been studied in children with those tumours since 1990 in JAPAN.

The histology of the primary tumour was mandatory to allow diagnosis of the neuroblastoma according to the International Neuroblastoma Pathology Classification, with the central review system by the Committee of Japanese Pediatric Tumor Pathology since 1994.

2.1. Statistical analysis

The Kaplan and Meier product limit methods were used to estimate the event-free survival (EFS) and the over-all survival (OS). The EFS calculated from diagnosis to the first event; relapse, progression or death (exception of other reason death). OS is calculated from diagnosis to death, excluded other reason death. Because the number of the events of each group was very small, we omitted the other reason death not to make bias. The Cox proportional hazards model was used to estimate the hazard ratios (HRs) and 95% confidence intervals (CI).The exact test from the permutation of the log-rank statistic was used to compare the EFS or OS probabilities between subgroups of patients. Differences between the two groups in categorical data were analysed by means of Fisher's exact probability test or the chi-square test. Two-sided *P*-values under 0.05 were considered as significant.

3. Results

The rates of stage 4s patients were 10.7%, 6.3% and 3.3% in patients ⩽11-months of age, 12–17 months of age, and ⩾18 months of age, respectively.

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Stage 4s patients frequently present even at 12 months of age and older, although their frequency decreases with age. Since 1985, the high numbers of patients under 11 months of age is the reason why cases detected by screening are included. However, there is no difference in the frequency of patients detected by screening between stage 4 and 4s groups (Table 1).

The rates of *MYCN* amplified stage 4s patients were 3.7%, 0% and 0% in those ⩾11 months of age, 12–17-months of age and ⩾18 months of age, respectively. The rates of *MYCN* amplified patients were smaller in the stage 4s groups than in the stage 4 groups in each group ($P < 0.001$, $P = 0.04$ and $P < 0.001$, respectively). Similarly, the rates of patients with unfavourable histology were smaller in the stage 4s groups than in the stage 4 groups. However, the difference in the frequency of patients with unfavourable histology between the two stage groups was not significant in those 12–17-months of age, because of the small number of patients. The stage 4s patients displayed a lower mean serum LDH value than the stage 4 patients in each group (Table 1).

In stage 4s patients ⩽11-months old, observation and surgery alone were 6.2% and 6.9%, respectively. Infants less than 12-months old were treated with a different protocol between stage 4s and 4 group. Those stage 4s patients received less dose chemotherapy than stage 4. Patients >12-months of age with stage 4s and 4 tumour received the same induction chemotherapy and most of them received surgical resection. The other hand, the number of patients who received high-dose chemotherapy with stem cell transplantation were smaller in the stage 4s groups than in the stage 4 groups in each age category ($P = 0.002$ and $P = 0.017$, respectively), for patients ⩽11 months of age and 12–17-months of age. All patients ⩽11-months of age who received high dose chemotherapy have tumours with *MYCN* amplification. There are no patients who received surgical resection only or

Table 1
Characteristics of patients with INSS stage 4 or stage 4s neuroblastoma.

		⩽11 m			12–17 m			⩽18 m		
		No.	%	*P*	No.	%	*P*	No.	%	*P*
Patients	Total	2579			252			1003		
	Stage 4s	275	10.7		16	6.3		33	3.3	
	Stage 4	294	11.4		73	29.0		523	52.1	
Screening	Stage 4s	174	63.2							
	Stage 4	154	52.4	0.173						
MNA/no-MNA	Stage 4s	8/206	3.7		0/9	0		0/32	0	
	Stage 4	30/156	16.1	<0.001	18/36	25	0.04	68/186	26.8	<0.001
UFH/FH	Stage 4s	4/95	5.3		0/5	0		4/23	14.8	
	Stage 4	11/78	12.4	0.014	8/16	33.3	0.129	78/97	44.6	0.003
LDH(U/L) (mean level)	Stage 4s	672.4			441.4			675.5		
	Stage 4	1483.8		<0.001	4755.6		<0.001	2316.4		0.019
Therapy										
Observation	Stage 4s	17	6.2		0	0		0	0	
Surgery alone		19	6.9		1	6.2		0	0	
Chemo+surgery		239	86.9		15	93.8		33	100	
Radiation		13	4.7		4	25		13	39.3	
HDT with SCT		4	1.5		0	0		5	15.2	
Observation	Stage 4	0	0		0	0		0	0	
Surgery alone		8	2.7		0	0		0	0	
Chemo+surgery		286	97.3	0.002	73	100	0.017	512	97.9	0.130
Radiation		90	30.6		23	31.5		162	31	
HDT with SCT		19	6.4		20	27.4		142	27.2	
Outcome										
Alive	Stage 4s	258	93.8		15	93.8		17	51.5	
Dead of disease		9	3.3		0	0		12	36.4	
Therapic death		5	1.8		0	0		2	6.1	
Other reason death		1	0.4		0	0		0	0	
Unknown		2	0.7		1	6.2		2	6.1	
Alive	Stage 4	215	73.1		19	26		112	21.4	
Dead of disease		39	13.3		44	60.3		351	67.1	
Therapic death		15	5.1		9	12.3		39	7.5	
Other reason death		3	1		0	0		6	1.1	
Unknown		22	7.5		1	1.4		15	2.9	

Abbreviations: MNA, MYCN amplification; UFH, unfavorable histology; FH, favorable histology; HDT, high dose therapy; SCT, stem cell transplantation.

Please cite this article in press as: Iehara T. et al., Is the prognosis of stage 4s neuroblastoma in patients 12 months of age and older really excellent? *Eur J Cancer* (2012), doi:10.1016/j.ejca.2012.01.010

observation in stage 4 and 4s patients aged ⩾18-months. In other words, stage 4s patients aged <18-months received less intensive treatment than stage 4 patients.

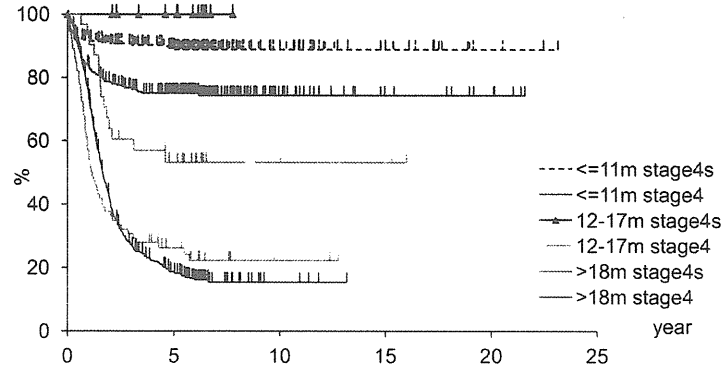
The details of prognosis of each groups were described in Table 1.The prognoses of stage 4s patients were good. Especially, the 5-year overall and event-free survival rates of the cases ⩽11 months of age, 12–17-months of age were excellent (91.2/89.4% and 100/100%, respectively) (Figs. 1 and 2). Comparing stage 4s with stage 4 in the same age, it was found that groups of stage 4s had a significantly better prognosis than the stage 4 groups (Figs. 1 and 2). For example, the *P*-values of the event-free survival rates were 0.004, 0.006 and <0.001, in patients ⩾11 months of age, 12–17-months of age and ⩾18 months of age, respectively.

4. Discussion

After Evans and DAngio reported on the uniqueness of stage 4s tumours concerning their spontaneous regression, most stage 4s tumours have been considered to be low risk tumours with an excellent prognosis.[1,2] Although there has been one report that stage 4s neuroblastoma patients do not have a poor prognosis even with *MYCN* amplification,[6] other reports have reported a poor prognosis in patients with stage 4s tumours with unfavourable prognostic factors such as *MYCN* amplification.[7–9] However, the tumours with poor prognostic factors are few in stage 4s neuroblastoma; the Children's Cancer Group Study reported that *MYCN* amplified tumour represented 0% of 80 stage 4s tumours,[10] and *MYCN* amplified cases constituted only 6% in 94 cases with stage 4s from the French Society of Pediatric Oncology.[11] In addition, it was reported that only 3.8% of all stage 4s tumours show an unfavourable histology.[10] From our results, only eight cases (3.7%) with stage 4s tumours showed *MYCN* amplification, and a few cases showed an unfavourable histology.

Presently, a few cases with unfavourable prognostic factors were evident in stage 4s cases involving infants both ⩽11 months of age and ⩾12 months of age. In the stage 4s patients, the serum LDH level was lower than the stage 4 patients in each group. These mean that the stage 4s neuroblastoma cases were less aggressive than the stage 4 cases at all ages were. These result that stage 4s patients who are ⩾12 months of age should have a better prognosis than stage 4 patients have, and these stage 4s cases should be different from stage 4 cases. Recently, some studies were conducted to clarify the biological difference between stage 4s and stage 4 using microarray analyses.[12,13] Although these studies did not discriminate between these stages in terms of genomic abnormalities, the possibility of a relationship between some partial chromosomal aberration, such as 17q, and clinical behaviour has been suggested.[13]

According to our research, the ratio of the stage 4s cases decreased and those of stage 4 increased with increasing age. The following two hypotheses can be suggested to the reason why the incidence of stage 4s cases changes with age. Firstly, stage 4 tumours, which are different from stage 4s tumours, developed with advancing age. The different biological characteristics of stage 4s and stage 4 tumours support this view. The number of cases detected might clinically decrease, because stage 4s tumours show spontaneous regression.[14–16] The second hypothesis assumes that stage 4s



	N	5y-EFS	
< = 11m stage 4s	273	89.4%	HRs;1.17
< = 11m stage 4	272	75.0%	95%CI;1.65-3.14, *P*=0.004
12-17m stage 4s	15	100%	HRs;3.57
12-17m stage 4	72	26.1%	95%CI;2.81-4.52, *P*=0.006
> = 18m stage 4s	31	53.1%	HRs;2.71
> = 18m stage 4	508	18.7%	95%CI;1.59-4.62, *P*<0.001

Fig. 1. Comparison of event-free survival rates of between stage 4s and stage 4 in patients <11 months old, ⩾12 to ⩽17 months, and ⩾18 months old. The event-free survival rates; The groups of stage 4s had better prognosis than the groups of stage 4 in patients <11 months old, ⩾12 to ⩽17 months, and ⩾18 months old (hazard ratios; 1.17, 3.57 and 2.71, $P = 0.001$, $P = 0.006$ and $P < 0.001$, respectively).

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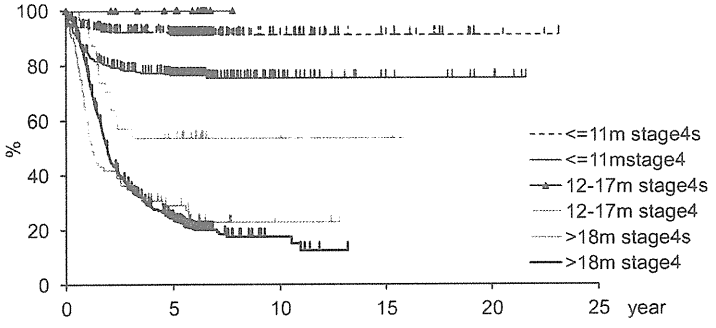

	N	5y-OS	
< = 11m stage 4s	273	91.2%	HRs;1.06 95%CI;1.79-4.64, P<0.001
< = 11m stage 4	272	76.3%	
12-17m stage 4s	15	100%	HRs;3.56 95%CI;2.67-4.63, P=0.007
12-17m stage 4	72	28.9%	
> = 18m stage 4s	31	53.5%	HRs;2.38 95%CI;1.40-4.06, P=0.001
> = 18m stage 4	508	24.4%	

Fig. 2. Comparison of overall survival rates of between stage 4s and stage 4 in patients <11 months old, ⩾12 to ⩽17 months, and ⩾18 months old. The overall survival rates; The groups of stage 4s had the better prognosis than the groups of stage 4 in patients <11 months old, ⩾12 to ⩽17 months, and ⩾18 months old (hazard ratios; 1.06, 3.56 and 2.38, $P < 0.001$, $P = 0.007$ and $P = 0.001$, respectively).

tumour change into stage 4 tumours, thus acquiring malignancy and thereby inducing clonal evolution.

However, it has been reported in a small number of cases that stage 4s tumours that progress to stage 4 tumours ultimately die.[17–19]

Presently, the cases with stage 4s tumours displayed a better prognosis than those cases with stage 4 tumour in infants aged ⩾12 months. Especially, the stage 4s cases aged 12–17 months had a good prognosis (100% 5 year event-free survival rate). According to the report of other countries, cases involving infants ⩾12 months of age with metastatic disease are now classified into stage 4 and receive intensive treatment.[20–22] In our study, cases ⩾12-months of age with stage 4s and 4 tumours were treated with the same induction chemotherapy consisting of cyclophosphamide and pirarubicin, cisplatin, vincristine or etoposide. The number of patients who received high-dose chemotherapy with stem cell transplantation was smaller in the stage 4s groups than in the stage 4 groups in aged 12–17-months category. As these stage 4s cases from 12–17-months of age were previously defined high risk group, they should now receive less intensive chemotherapy. It has been reported that the patients from 12–18 months of age with stage 4 non-amplified *MYCN* neuroblastoma have a better prognosis than older children.[20,21] This group may include stage 4s cases from 12 to 17 months of age. These cases are appropriate as a low risk group as well as the cases aged ⩽11 months. Therefore, the concept of stage MS of INRG of patients <18 months of age is proper.

On the other hand, the 5-year overall and event-free survival rates of stage 4s patients aged ⩾18-months were not so good (namely, 53.1% and 53.5%, respectively). This group should therefore be classified as belonging to a high risk group, and the initial intensive treatment should not be reduced.

Conflict of interest statement

None declared.

Acknowledgements

We would like to sincerely thank all patients with neuroblastoma and their families, paediatric oncologists and paediatric surgeons in Japan. We also thank the Japanese Society of Pediatric Oncology and Japanese Society of Pediatric Surgeons. This project is supported by Grants-in Aid for Scientific Research (16-Kodomo-012) from the Ministry of Health, Labor, and Welfare of the Government of Japan.

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Genomics 98 (2011) 120–127

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Methylation dynamics of IG-DMR and *Gtl2*-DMR during murine embryonic and placental development

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ARTICLE INFO

Article history:
Received 5 December 2010
Accepted 12 May 2011
Available online 18 May 2011

Keywords:
Genomic imprinting
DNA methylation
IG-DMR
Gtl2-DMR

ABSTRACT

The *Dlk1-Dio3* imprinted domain on mouse chromosome 12 contains IG-DMR and *Gtl2*-DMR, whose methylation patterns are established in the germline and after fertilization, respectively. In this study, we determine that acquisition of DNA methylation at the paternal allele of the *Gtl2*-DMR is initiated after the blastocyst stage and completed by embryonic day 6.5, and that *Gtl2* (approved symbol: Meg3) is monoallelically expressed from the maternal allele as early as the blastocyst. Therefore, DNA methylation at the *Gtl2*-DMR is not a prerequisite for the imprinted expression of *Gtl2*, which may be involved in the control of proliferation and differentiation of cells during early gestation. We also reveal that a subregion of the IG-DMR exhibits tissue-specific differences in allelic methylation patterns. These results add to the growing body of knowledge elucidating the mechanism whereby parent-of-origin-dependent DNA methylation at the IG-DMR leads to the imprinted expression of the *Dlk1-Dio3* cluster.

1. Introduction

Genomic imprinting is an epigenetic mechanism that regulates transcription, whereby the expression of a subset of genes is limited to or biased towards one parental allele. To date, over one hundred imprinted genes have been identified in the mouse (http://www.har.mrc.ac.uk/research/genomic_imprinting). Imprinted genes tend to be clustered on the genome. One of the common features among imprinted loci is that such genomic intervals include one or more differentially methylated regions (DMRs), which exhibit parent-of-origin dependent DNA methylation patterns [1]. DMRs have been classified into two types according to the time at which their DNA methylation patterns are established. Primary (germline) DMRs harbor allelic DNA methylation inherited from the male or the female gamete. Secondary (post-zygotic) DMRs acquire parent-of-origin dependent methylation patterns after fertilization. In mice, germline DMRs are shown to be established during the oocyte growth stage (postnatal days 5 to 20) [2,3] or the prospermatogonia stage (embryonic days 14.5 to newborn) [4–6] by a DNMT3L-dependent mechanism [7–12]. Several germline DMRs have been shown to govern the imprinted expression of genes as well as the methylation of post-zygotic DMRs within chromosomal regions. These germline DMRs, known as imprinting control regions (ICRs), regulate these regions by *cis*-acting mechanisms. [13–15]. On the other hand, little is known about the function of secondary DMRs in the regulation of imprinted gene expression, as well as *cis*-acting mechanisms and *trans*-acting factors that establish DNA methylation at secondary DMRs.

Studies focusing on the regulatory functions of ICRs have revealed a number of different molecular mechanisms that underlie the coordinated and long-range regulation of imprinted genes. In the *H19/Igf2* domain, long range chromatin interactions mediated by CTCF between the primary *H19*-DMR and the secondary *Igf2*-DMRs play an integral role in the regulation of imprinted gene expression at this locus [13,16]. Another mechanism involves non-coding (nc) RNAs such as *Airn* in the *Igf2r* locus and *Kcnq1ot1* in the *Kcnq1* imprinted gene cluster. These ncRNAs are transcribed from ICRs and are shown to be functionally linked to the silencing of genes in *cis* through gene- and lineage-specific repressive chromatin modifications [14,17,18]. These two mechanisms are likely to be involved in the regulation of many other imprinted loci as well. However, the sequence of events leading to the establishment and maintenance of imprinted expression for a cluster of genes remains largely elusive for many imprinted loci.

The *Dlk1-Dio3* imprinting cluster on mouse distal chromosome 12 contains the intergenic germline-derived DMR (IG-DMR) and the *Gtl2*-DMR, whose methylation patterns are established in the germline and after fertilization, respectively [19,20]. The cluster consists of at least three paternally expressed protein-coding genes (*Dlk1*, *Rtl1*, and *Dio3*), and four maternally expressed ncRNAs (*Gtl2*, *Anti-Rtl1*, *Rian* and *Mirg*). The IG-DMR is shown to function as the ICR of this imprinted gene cluster [15,21]. A targeted disruption study of the IG-

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doi:10.1016/j.ygeno.2011.05.003

DMR [15] has revealed that the maternally inherited IG-DMR, which is unmethylated, is essential in the embryo to maintain the unmethylated status of the *Gtl2*-DMR, the expression of the ncRNAs, and the repression of the protein-coding genes, on the maternal allele. However, the principal mechanism whereby the allele-specific methylation at the IG-DMR leads to the imprinted expression of the cluster of genes on chromosome 12 is unknown. It has been also demonstrated that, in the placenta, the absence of the maternally inherited IG-DMR results in the activation of protein-coding genes but only partial repression of the ncRNAs, and leads to no phenotypic consequence [21]. Therefore, mechanisms underlying the imprinted expression of the maternally-expressed ncRNAs are different between the embryonic and the extra-embryonic tissue lineages.

Among known secondary DMRs, the *Gtl2*-DMR is unique in that it has been demonstrated to possess an essential long-range imprinting regulatory function. A neonatal patient showing a paternal uniparental disomy 14-like phenotype in the body but not in the placenta was identified to have a maternally-inherited heterozygous microdeletion that encompasses the *MEG3*-DMR (the human orthologue of the mouse *Gtl2*-DMR) but not the IG-DMR. In this patient, the maternal allele of *DLK1* has been shown to be reactivated [22]. Recent studies have used knockout mouse models with targeted deletions of the *Gtl2* locus, spanning the *Gtl2*-DMR. These studies have also suggested that *Gtl2* and/or *Gtl2*-DMR could regulate the expression of maternally expressed genes, indicating that the methylation of the *Gtl2*-DMR is a critical element in the *Dlk1-Dio3* imprinted domain [23,24]. In light of the critical roles that *Gtl2* and *Gtl2*-DMR may play in the imprinted regulation of this region, understanding the epigenetic mechanisms that govern them during early development is expected to further elucidate the mechanisms regulating the *Dlk1-Dio3* imprinted domain.

Recently, Stadtfeld et al. [25] reported that mouse induced pluripotent stem cells (iPSC) with repressed expression of maternally expressed ncRNAs in the *Dlk1-Dio3* domain contributed poorly to chimeras and failed to generate all-iPSC mice. In contrast, iPSCs with normal ncRNA expression patterns contributed to high-grade chimeras and produced all-iPSC mice. Hypermethylation of both the IG-DMR and the *Gtl2*-DMR was found to be associated with the reduced expression of ncRNAs in the iPSCs exhibiting poor contribution to chimeras [25]. This epimutation is considered to be caused by the iPSC reprogramming, rather than existing aberrant methylation patterns in the DMRs of the somatic cell of origin [25]. Therefore, a better understanding of the epigenetic regulation of these DMRs may eventually lead to improved reprogramming strategies of iPSC.

In this study, we determined the allelic DNA methylation patterns at the IG-DMR and the *Gtl2*-DMR, as well as the allelic expression patterns of *Dlk1* and *Gtl2* at early developmental stages (embryonic days 3.5 to 7.5) in embryonic and extra-embryonic tissues.

2. Results

2.1. Developmental dynamics of allelic DNA methylation patterns at IG- and Gtl2-DMRs in sperm, blastocysts, and post-implantation embryos

We examined allelic DNA methylation patterns at the IG-DMR and the *Gtl2*-DMR in whole embryos at embryonic day 3.5 (E3.5) and E5.5 as well as their methylation status in sperm. We regarded the genomic intervals defined by Kobayashi et al. [26] and Takada et al. [20] as the IG-DMR and the *Gtl2*-DMR, respectively. Three regions within the IG-DMR and the two regions within the *Gtl2*-DMR were chosen as targets for bisulfite sequencing (Fig. 1A). All five regions contain at least one single nucleotide polymorphism (SNP) between C57BL/6 (B6) and JF1/Ms (JF1) strains that can distinguish parental alleles in F1 hybrid materials (see details in Section 4.2 in the Materials and methods).

The IG-DMR was heavily methylated in all three regions (methylation percentage 81.3–95.8%) in sperm (Fig. 1B) as shown previously [20]. In blastocysts (E3.5), all three regions within the IG-DMR were maternally unmethylated (0–1.8%), yet paternally methylated (43.1–71.8%) (Fig. 1B). The observed levels of paternal methylation at E3.5 were significantly lower than those observed in sperm, implying that the paternal IG-DMR partially loses methylation at CpG dinucleotides after fertilization. This loss of methylation may be caused by the active and the passive demethylation of the paternal genome in pronucleus and preimplantation embryos, respectively [27,28]. At E5.5, the maternal allele of IG-DMR was found to be hypomethylated (1.9–18.3%), and the paternal allele to be hypermethylated (80.2–91.4%; Fig. 1B). The methylation level of the paternal allele was consistently higher at E5.5 than at E3.5. Additionally, it was almost fully methylated at E5.5 in all three regions examined, suggesting that de novo methylation events occur on the paternal allele of the IG-DMR during the developmental period between E3.5 and E5.5. These are the first results to illustrate the developmental dynamics of paternal methylation levels at the IG-DMR around the implantation period.

The differential methylation of the *Gtl2*-DMR on the paternal allele has been shown to be established in E13.5 embryos [20]. However, the post-zygotic stages at which the region's paternal methylation is initiated and completed remain unknown. Additionally, the relationship between the imprinted expression of *Gtl2* and DNA methylation at the *Gtl2*-DMR has not been elucidated. We confirmed that the *Gtl2*-DMR was unmethylated in sperm, and found that it was unmethylated on both parental alleles in blastocysts (Fig. 1B). In E5.5 embryos, the maternal allele remained hypomethylated (6.0 and 11.7%), while the paternal allele became partially methylated (55.2% in R4 and 42.7% in R5 regions) (Fig. 1B). In E6.5 and E7.5 embryos, the paternal allele of the *Gtl2*-DMR was found to be heavily methylated (75.8% or higher) (Fig. 1C). These data demonstrate that paternal methylation of the *Gtl2*-DMR is initiated after the blastocyst stage and is completed by E6.5 stage in the embryonic lineage.

2.2. Allelic DNA methylation patterns at IG- and Gtl2-DMRs in early and late gestational stages

It has been reported that, in both human and mouse placenta, the IG-DMR maintains its allele-specific methylation patterns, whereas the *Gtl2*-DMR does not show differential methylation between parental alleles [21,29,30]. To determine the developmental stage at which the allelic methylation patterns at the *Gtl2*-DMR diverge between embryonic and extra-embryonic lineages, we examined the DNA methylation status of the *Gtl2*-DMR as well as the IG-DMR in E6.5 and E7.5 tissues. In extra-embryonic tissues at both E6.5 and E7.5 stages, the *Gtl2*-DMR was partially methylated on both parental alleles, whereas its differential methylation was well maintained in embryonic tissues (Fig. 1C). The *Gtl2*-DMR was previously shown to be partially methylated on both parental alleles in late gestation (E16.5) placentas [21,29]. Our results demonstrate that the allelic methylation pattern observed in E16.5 placenta is already present in the extra-embryonic lineage at E6.5 stage.

Unexpectedly, we observed loss of differential methylation at the R2 and R3 regions within the IG-DMR in extra-embryonic tissues, although the R1 region maintained its differential methylation. The loss of differential methylation was more evident in E7.5 stage than in E6.5 stage (Fig. 1C). To assess whether the loss of differential methylation at the R2/R3 regions as well as the R4/R5 regions was specific to the extra-embryonic lineage, we examined the allelic methylation patterns of the R1–R5 regions in fetal tissues from late gestation time points. E16.5 skeletal muscle, E15.5 brain, and E16.5 liver were analyzed since they represent tissues derived from the

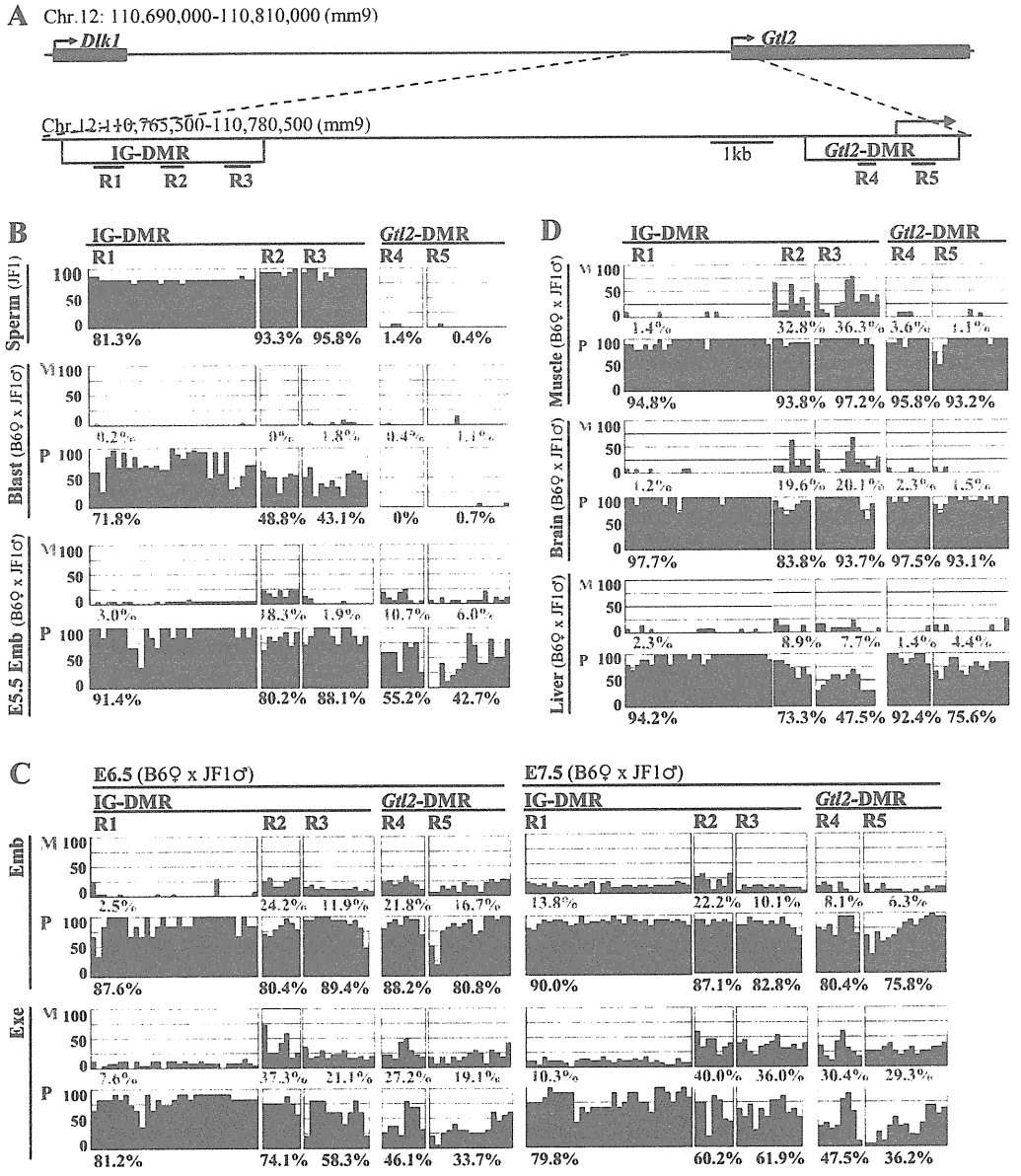

Fig. 1. Allelic DNA methylation patterns at the IG-DMR and the *Gtl2*-DMR during embryonic and extra-embryonic development. (A) A schematic diagram of the locus containing the IG-DMR and the *Gtl2*-DMR (shown as open boxes). The bars under the open boxes indicate the regions (R1–R5) analyzed by bisulfite sequencing. The arrow indicates the transcription start site of *Gtl2*. Scale bar = 1 kb. (B–D) Graphical representation of the methylation percentage at each CpG site in sperm, blastocysts at E3.5 (Blast), and embryos at E5.5 (E5.5_Emb) (B), in embryonic (Emb) and extra-embryonic (Exe) tissues at E6.5 and E7.5 (C), and in fetal tissues (E16.5 skeletal muscle, E15.5 brain, and E16.5 liver) (D). The vertical bars represent the percentage ratio of methylated cytosine at each CpG site, which were determined from the data of clone-based bisulfite sequencing (Supplementary Fig. 1). Overall methylation percentage for each region (the number of methylated CpGs per the number of total CpGs) is shown under each panel. As described in the Materials and Methods (Section 4.2), methylation percentage for each CpG site and each region was calculated using bisulfite sequencing data for a single sample (sperm and E15.5/16.5 fetal tissues) or two independent samples (E3.5 to E7.5 samples). M and P denote maternal and paternal alleles, respectively.

mesoderm, the ectoderm, and the endoderm, respectively. We found that differential methylation at the R1 region of the IG-DMR and the R4/R5 region of the *Gtl2*-DMR was strictly conserved. In contrast, differential methylation at the R2/R3 regions of the IG-DMR was partially lost to varying degrees in these tissues (Fig. 1D). Partial gain of methylation on the maternal allele (most notably observed in skeletal muscle) and partial loss of methylation on the paternal allele (most remarkably observed in the liver) were detected. Taken together, our data demonstrate that only a subregion of the IG-DMR containing the R1 region strongly maintains allele-specific differential methylation during embryonic development, whereas the rest of region containing the R2/R3 regions exhibits various tissue-specific allelic methylation patterns.

2.3. Allelic expression patterns of Dlk1 and Gtl2 during embryonic and extra-embryonic development

We subsequently assessed the expression levels and the allelic expression patterns of *Dlk1* and *Gtl2*. Although the expression of *Gtl2* is shown to be detectable as early as the pre-implantation stage [31], previous studies have not assessed the expression levels of these genes in a quantitative manner and have not determined their allelic expression patterns during early gestation (E3.5 to 7.5). Therefore, we performed both quantitative RT-PCR and pyrosequencing to quantify the allelic expression of these transcripts.

To determine the relative expression levels of *Gtl2* and *Dlk1*, quantitative RT-PCR was performed using E3.5 to E7.5 tissues and

E16.5 skeletal muscle tissues. The E16.5 skeletal muscle was chosen as a reference tissue for the relative expression levels of *Dlk1* and *Gtl2* because both transcripts have been shown to be highly expressed in E14.5 to E18.5 skeletal muscle [19,20,31]. We obtained the Ct (cycle threshold) values of *Gtl2* in E5.5–7.5 tissues (23.2 to 33.5) within the Ct value range of the standard curve for *Gtl2* (20.0 to 35.3), and determined the relative expression levels of *Gtl2* in these tissues, ranging from 0.0036 (E7.5Exe) to 0.31 (E6.5Exe) relative to the average level of E16.5 skeletal muscle samples (Fig. 2A). As demonstrated by Schuster-Gossler et al. [31], we were consistently able to detect the expression of *Gtl2* in blastocysts in our replicate samples, and determined its average relative expression level to be 0.011. However, this value should be considered with caution because the Ct values of the blastocyct samples (35.9, 36.4, and 36.5) were slightly out of range. Our results demonstrate that *Gtl2* is expressed at low levels at E3.5, up-regulated transiently at E5.5 and E6.5, and down-regulated at E7.5. Variation in the relative expression levels of *Gtl2* among three replicate samples was most remarkable in extra-embryonic tissues at E6.5. Because *Gtl2* expression seems to decrease rapidly between E6.5 and E7.5 in extra-embryonic tissues, subtle differences in the developmental stage among our E6.5 samples may account for the variation in *Gtl2* expression levels.

We obtained the Ct values of *Dlk1* in E5.5–7.5 tissues (26.5 to 35.4) within the Ct value range of the standard curve for *Dlk1* (21.6 to 36.8), and determined that the relative expression levels of *Dlk1* in these tissues ranged from 0.00021 (E7.5Exe) to 0.015 (E7.5Emb). The expression of *Dlk1* was undetectable in the blastocyst samples, and was consistently low in E5.5–7.5 tissues. In embryonic tissues, *Dlk1* expression levels tended to increase as embryonic development progressed (Fig. 2A). These results represent the first quantitative measurement of *Gtl2* and *Dlk1* expression levels during early gestational stages. The transient up-regulation of *Gtl2* around E5.5 and E6.5 stages suggest a possible role of this ncRNA in the control of growth and differentiation at these developmental stages.

To complete our analysis of the allelic expression patterns of *Glt2* and *Dlk1*, we quantitatively measured the allelic expression levels of these transcripts by pyrosequencing. *Gtl2* was consistently found to be

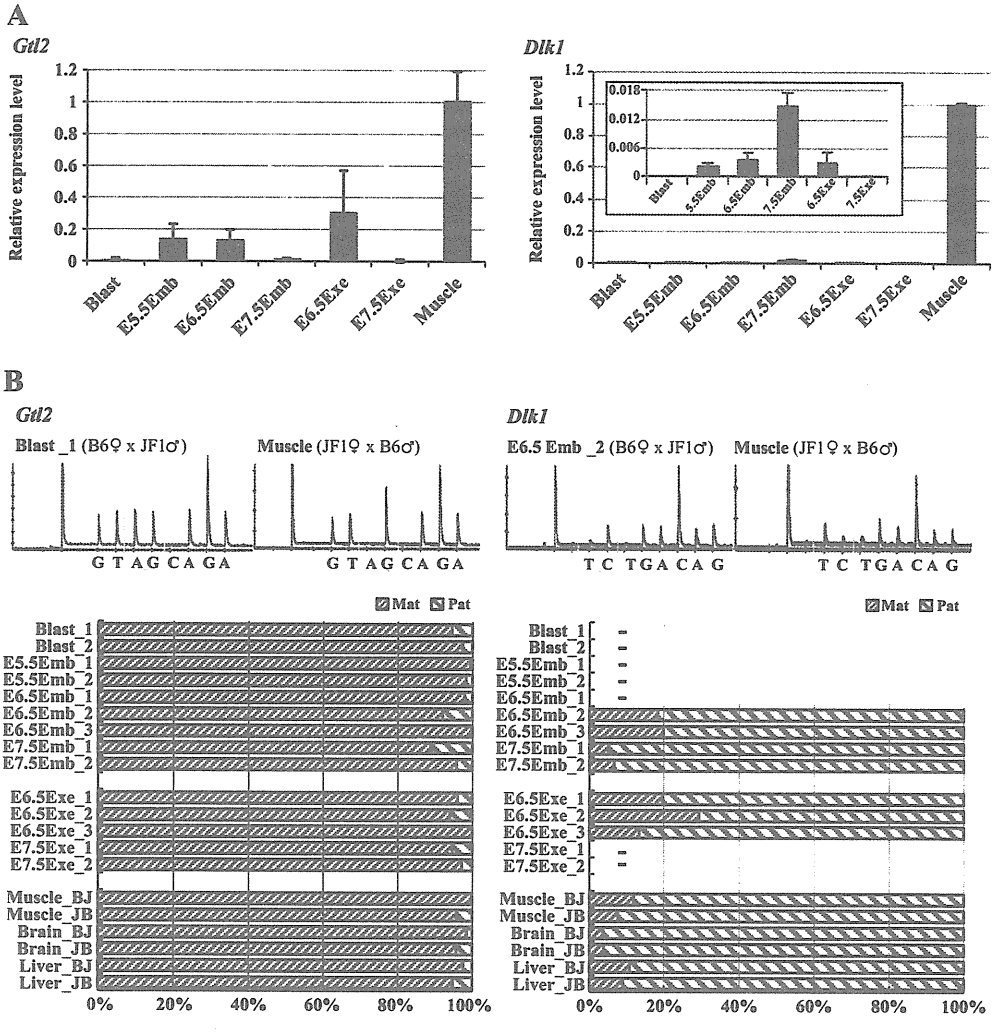

Fig. 2. Expression analysis of *Gtl2* and *Dlk1* during embryonic and extra-embryonic development. (A) Graphical representation of the relative expression levels of *Gtl2* and *Dlk1* in embryonic and extra-embryonic tissues at E3.5 to E7.5. The bars represent the mean expression levels of replicate samples relative to the mean expression level of E16.5 muscle samples (n = 4). Error bar = standard deviation (SD). The mean ± SD of each sample set for *Gtl2*: Blast, 0.011 ± 0.0094; E5.5Emb, 0.14 ± 0.088; E6.5Emb, 0.14 ± 0.061; E7.5Emb, 0.018 ± 0.0033; E6.5Exe, 0.31 ± 0.26; E7.5Exe, 0.0036 ± 0.0095. The mean ± SD for *Dlk1*: E5.5Emb, 0.0023 ± 0.00066; E6.5Emb, 0.0035 ± 0.0016; E7.5Emb, 0.015 ± 0.0025; E6.5Exe, 0.0030 ± 0.0021; E7.5Exe, 0.00021 ± 0.00015. (B) Quantitative allelic expression analysis of *Gtl2* and *Dlk1* by pyrosequencing. The top panels show examples of pyrograms. The yellow box in each pyrogram denotes the peaks at SNPs between the B6 and the JF1 strains. The alleles (B6/JF1) of SNPs are A/G for *Gtl2* and T/C for *Dlk1*. The bottom panels represent the allelic expression ratios of the paternal (Pat, blue stripe) and the maternal (Mat, red stripe) alleles. "–" in the panel for *Dlk1* indicates that the corresponding sample was not analyzed due to the absence or low expression of *Dlk1*.

maternally expressed in blastocysts (E3.5) and E5.5 embryos, as well as latter developmental stages (E6.5 and E7.5) in both embryonic and extra-embryonic tissues (Fig. 2B). These results demonstrate that the repression of *Gtl2* on the paternal allele occurs at E3.5 without methylation at its promoter (R4/R5) (Fig. 1B). Due to the relatively low levels of *Dlk1* expression, we only determined the allelic expression status of *Dlk1* in embryonic tissues at E6.5 and E7.5 and extra-embryonic tissues at E6.5. *Dlk1* was predominantly expressed from the paternal allele in E6.5 tissues, and this allelic preference was more dramatic in embryonic tissues at E7.5 (Fig. 2B). Relaxation of imprinting was more evident for *Dlk1* than *Gtl2* in the E6.5 tissues. Using a quantitative method, we successfully determined the allelic expression patterns of *Gtl2* and *Dlk1* during early gestational stages for the first time.

We also measured allelic expression levels of *Gtl2* and *Dlk1* in fetal skeletal muscle, brain, and liver tissues at E15.5/E16.5, and found that *Gtl2* and *Dlk1* were exclusively or predominantly expressed from the maternal and the paternal alleles, respectively (Fig. 2B). The ratio of *Dlk1* expression from the maternal allele (relaxation of imprinting) was higher in muscle and liver than in brain. Such allelic expression patterns replicate the data produced by da Rocha et al. [32]. We compared allelic methylation patterns at the R2 and R3 regions of IG-DMR (Fig. 1D) with the allelic expression patterns of *Dlk1* (Fig. 2B) in three tissues. However, there was no clear correlation between methylation levels at the R2/R3 regions and the extent to which *Dlk1*'s imprinting was relaxed.

3. Discussion

3.1. Establishment of allele-specific DNA methylation patterns at secondary DMRs

In this study, we demonstrated that the paternal allele of the *Gtl2*-DMR gains DNA methylation after the blastocyst stage and becomes fully methylated by the E6.5 stage in the embryonic lineage. Additionally, we determined that DNA methylation at the *Gtl2*-DMR is not a prerequisite for the imprinted expression of *Gtl2* in early development (summarized in Fig. 3 and Table 1). The timing of the establishment of parent-of-origin-dependent differential methylation patterns during post-zygotic development has previously been determined for several secondary DMRs [13,33–38] (summarized in Table 1). Although the developmental stage at which differential methylation is established differs among secondary DMRs including the *Gtl2*-DMR, it is frequently observed that the imprinted expression of the gene associated with the secondary DMR is already established prior to the gain of differential DNA methylation. This observation holds true even among DMRs which are regulated by different mechanisms.

In mid-to-late gestation fetuses that carry an insertion mutation upstream of the *Gtl2*-DMR on the paternal allele, it has been observed that the *Gtl2*-DMR loses its paternal methylation and *Gtl2* is biallelically expressed [39,40]. It has also been shown that *Cdkn1c* is biallelically expressed in E9.5 embryos that are deficient in DNMT1 activity [33]. These observations suggest that paternal methylation of the *Gtl2*- and the *Cdkn1c*-DMRs is necessary to maintain silencing of the paternal allele of these genes. However, whether DNA methylation plays a critical role in the maintenance of imprinted gene expression remains unproven for the other secondary DMRs. While Lsh, a member of the SNF2 family of chromatin remodeling proteins, is shown to be required for the proper acquisition of the paternal methylation at the *Cdkn1c*-DMR [41], information regarding such *trans*-acting factors is lacking for the majority of secondary DMRs.

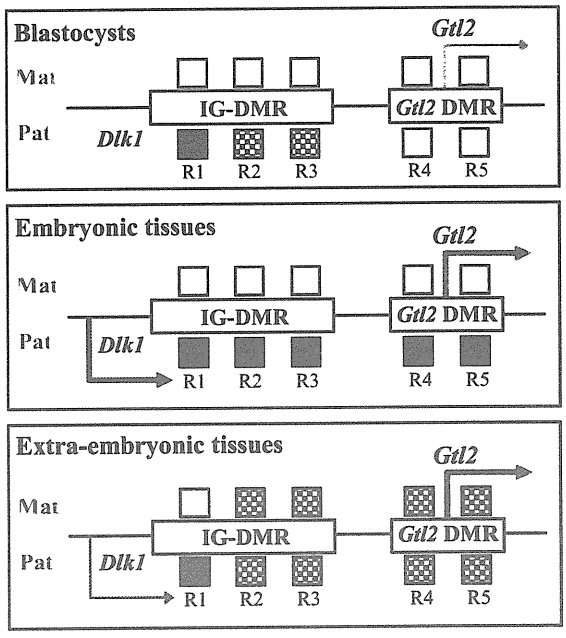

Fig. 3. Summary of the allelic DNA methylation patterns of IG-DMR and *Gtl2*-DMR and the allelic expression patterns of *Gtl2* and *Dlk1* during embryogenesis. The methylation and expression patterns in blastocysts and embryonic/extra-embryonic tissues at E6.5 and E7.5 are schematically shown. The squares above and below the DMRs symbolize the regions subjected to bisulfite sequencing analysis (R1 to R5). Open, filled, and mosaic squares indicate hypomethylated, hypermethylated, and intermediately methylated regions, respectively. Thick and thin arrows represent high and low expression levels of *Gtl2* and *Dlk1*. In blastocysts, the maternal allele-specific expression of *Gtl2* occurs without the paternal allele-specific methylation of the *Gtl2*-DMR. In embryonic tissues, the paternal allele-specific methylation of *Gtl2*-DMR is established by E6.5. Allele-specific differential methylation is well maintained in both the IG-DMR and the *Gtl2*-DMR in embryonic tissues. In extra-embryonic tissues at E6.5, imprinted expression of *Gtl2* and *Dlk1* occurs despite the loss of allele-specific differential methylation at the R2 and R3 regions in the IG-DMR and in the *Gtl2*-DMR. These allelic methylation/expression patterns suggest that the R1 region may contain a methylation-dependent element that is critical for the ICR function of the IG-DMR, and suggest that epigenetic modifications other than DNA methylation may play more critical roles in the maintenance of imprinted expression in the *Dio3-Dlk1* domain in extra-embryonic tissues than in the embryonic tissues.

3.2. Epigenetic modifications of the IG-DMR and the Gtl2-DMR during early development

In E7.5 embryo deficient in the Polycomb group (PcG) gene *Eed*, it has been shown that *Gtl2* is biallelically expressed whereas *Dlk1* is properly imprinted (paternally expressed) [42], suggesting a role for PcG complex proteins in the regulation of paternal *Gtl2* silencing. The EED-containing Polycomb Repressive Complex 2 (PRC2) catalyzes trimethylation of histone H3 lysine-27 (H3K27me3), which is a binding site for the repressive PRC1 complex [43]. It has been demonstrated by Hammoud et al. that, in human sperm, the *MEG3/GTL2*-DMR bears both active trimethylation of histone H3 lysine-4 (H3K4me3) and repressive H3K27me3 histone marks [44]. DNMT3L recognizes unmethylated H3K4 and induces de novo DNA methylation by recruitment or activation of DNMT3A2 [11,12]. The interaction of DNMT3L with H3K4 is strongly inhibited by methylation at H3K4 (the higher the degree of methylation at H3K4, the more severely the binding of DNMT3L with histone H3 N-terminal is abolished) [11]. In light of the post-zygotic acquisition of DNA methylation at the *MEG3/GTL2*-DMR and the bivalent histone pattern in this region, Hammoud et al. hypothesized that H3K4me3 may prevent DNA methylation in the sperm and early embryo, and H3K27me3 may ensure early silencing at this locus [44]. In this study, we have shown that, in the blastocyst stage, *Gtl2* is already expressed primarily from the maternal allele in the absence of DNA