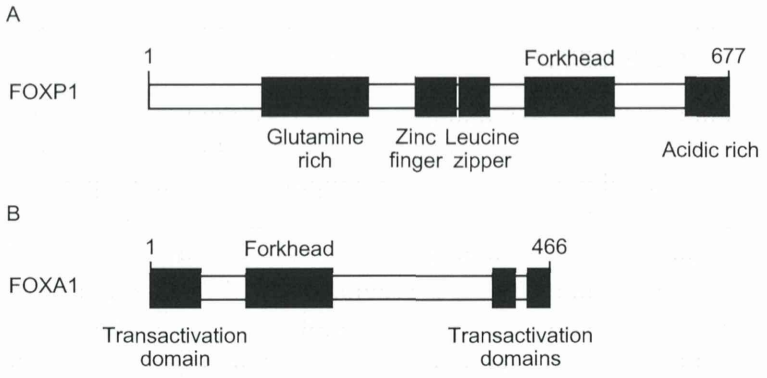

Figure 7.1 Schematic representation of the domain structure of forkhead box transcription factor FOXA1 and FOXP1 proteins. (A) FOXP1 structure. Forkhead domain is located in the C-terminal region of the FOXP1 protein. Zinc finger and leucine zipper domains, responsible for dimerization of FOXP1, are located in the central region. (B) FOXA1 structure. Forkhead domain is located in N-terminal region of FOXA1 protein. Three transactivation domains are located in both terminal regions.

(Fig. 7.1; Li, Weidenfeld, & Morrisey, 2004; Wang, Lin, Li, & Tucker, 2003). Recently, genome-wide studies with an aim of identifying ERα- and androgen receptor (AR)-binding sites have shown that FOXA1 plays a role in regulation of the nuclear receptor-mediated gene networks (Carroll et al., 2005, 2006; Lupien et al., 2008). FOXA1 is recognized as a pioneer transcription factor because binding of FOXA1 to chromatin DNA facilitates subsequent recruitment of ERα and AR to the genome (Grange, Roux, Rigaud, & Pictet, 1991; Lupien et al., 2008). Genome-wide mapping of ERα-, AR-, and FOXA1-binding events in breast and prostate cancer cells using high-throughput sequencing has further uncovered the involvement of several collaborative factors, including TLE1 and activator protein 2γ (AP-2γ), in the nuclear receptor-mediated transcription (Holmes et al., 2012; Tan et al., 2011). In breast cancer cells, several FOX family transcription factors may contribute to the ERα-mediated transcription by directly interacting with the ERα protein, as exemplified by FOXA1 and FKHR/FOXO1 (Carroll & Brown, 2006; Schuur et al., 2001).

Recent studies have shown that FOXP1 and FOXA1 play critical roles in estrogen signaling and in the biology of ERα-positive breast cancer (Ijichi et al., 2012; Shigekawa et al., 2011). These studies showed an upregulation in the expressions of *FOXP1* and *FOXA1* mRNAs induced by 17β-estradiol (E2) stimulation in ERα-positive MCF-7 cells. The

upregulation of both genes was observed in the early phase (3 h) after E2 stimulation, which suggests that both FOXP1 and FOXA1 are transcriptionally regulated by the estrogen in breast cancer cells. In addition, Giguère and his colleagues reported that estrogen upregulates the levels of FOXA1 protein 4–8 h after E2 stimulation (Laganière et al., 2005). Consistent with these findings, the findings of genome-wide chromatin immunoprecipitation (ChIP) analysis based on microarrays (ChIP-chip) showed three and two functional estrogen receptor-binding sites (ERBSs) within the *FOXP1* and *FOXA1* gene loci, respectively, in the genome of MCF-7 cells (Carroll et al., 2005). Conventional ChIP assay showed more than twofold enrichments of estrogen-dependent recruitment of ERα in these ERBSs, which suggested that the recruitment of ERα in the *FOXP1* and *FOXA1* loci contributes to the estrogen-dependent transcription of both *FOX* genes.

Further, FOXP1 and FOXA1 have been shown to serve as transcription factors that directly regulate the ERα-mediated transcription. Luciferase reporter analysis using a vector containing an estrogen-responsive element (ERE, ERE-tk-*luc*) showed that overexpression of either FOXP1 or FOXA1 significantly stimulated the ERα-mediated transactivation in MCF-7 cells in response to estrogen. siRNA-mediated knockdown of FOXA1 reduced ERα-mediated transactivation in the presence or absence of estrogen in MCF-7 cells. Consistent with these observations, the results of other studies showed upregulation of known estrogen-responsive genes, including *SHP* (Lai, Harnish, & Evans, 2003) and *LRH-1* (Annicotte et al., 2005), in FOXP1-overexpressing MCF-7 cells treated with estrogen. Similarly, the contribution of FOXA1 to ERα-mediated transcription was further confirmed by the FOXA1 siRNA-dependent reduction in estrogen-induced expressions of prototypic ERα target genes, progesterone receptor (*PgR*), and growth regulation by estrogen in breast cancer 1 (*GREB1*) (Ghosh, Thompson, & Weigel, 2000; Kastner et al., 1990). These observations suggest that both FOXP1 and FOXA1 stimulate ERα transcription activity in response to estrogen.

The mutual transcriptional regulations of *ERα* and *FOX* genes indicate that both FOXA1 and FOXP1 have the potential to promote estrogen-dependent proliferation of breast cancer cells. Moreover, FOXA1 also upregulates the migration of MCF-7 cells. These findings suggest that FOXP1 and FOXA1 regulate ERα in a positive feedback manner and play crucial roles in the estrogen-dependent cellular responses of ERα-positive breast cancer cells (Fig. 7.2).

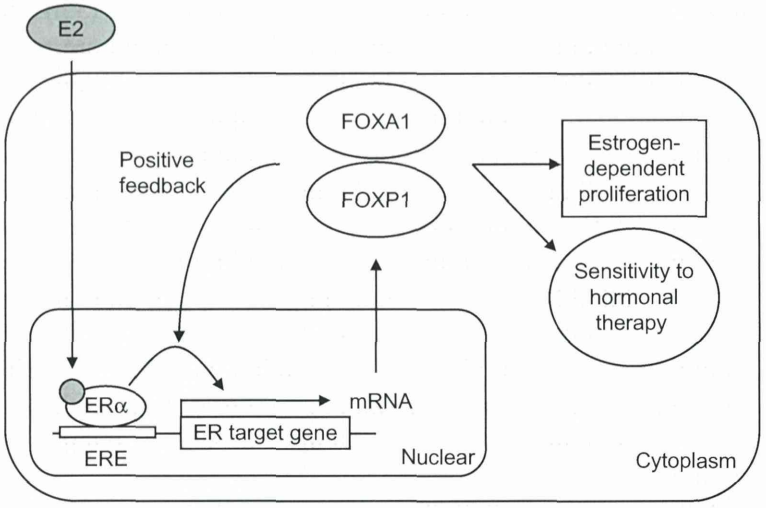

Figure 7.2 Model for cellular functions of FOXP1 and FOXA1 in estrogen signaling. *FOXP1* and *FOXA1* are primary target genes for estrogen receptor α (ERα) and regulate the ERα-mediated transcription in a positive feedback manner. FOXP1 and FOXA1 promote estrogen-dependent proliferation of breast cancer cells and contribute to the sensitivity to hormone therapy.

3. CLINICOPATHOLOGICAL SIGNIFICANCES OF FOXP1 AND FOXA1 IN ER-POSITIVE BREAST CANCER

Recent global gene expression studies on breast cancer have shown that high FOXA1 expression was positively correlated with the status of hormone receptors ERα and PgR and negatively correlated with histological grade and proliferation markers (Badve et al., 2007; Habashy et al., 2008; Thorat et al., 2008). In addition, FOXA1 expression was associated with better prognosis of cancer-specific survival, which indicated that FOXA1 can serve as a predictor for good prognosis of breast cancer (Badve et al., 2007; Habashy et al., 2008; Thorat et al., 2008; Wolf et al., 2007). On the basis of gene expression profiling studies, researchers have classified breast cancers into the following five intrinsic subtypes with unique molecular characteristics and prognostic significance (Perou et al., 2000; Sørlie et al., 2001): luminal A and B, HER2+/ERα−, basal-like, and normal-like subtypes. Luminal subtypes A and B are ERα-positive breast cancers, distinguishing subtype A from B by its higher levels of ERα and better prognosis of the patients (Sørlie et al., 2001). Among these subtypes, FOXA1

expression is best associated with luminal subtype A and FOXA1 immunoreactivity is shown as a significant predictor of cancer-specific survival for patients with ERα-positive tumors (Badve et al., 2007; Mehta et al., 2012). The prognostic relevance of FOXA1 in the breast cancers with relatively low risk will be useful for the determination of therapeutic methods (Badve et al., 2007; Thorat et al., 2008).

Altered expression of FOXP1 is associated with various types of tumors, including breast cancer (Banham et al., 2001, 2007; Barrans, Fenton, Banham, Owen, & Jack, 2004; Bates et al., 2008; Craig et al., 2011; Fox et al., 2004; Goatly et al., 2008; Hoeller, Schneider, Haralambieva, Dirnhofer, & Tzankov, 2010; Prown et al., 2008; Sagaert et al., 2006; Takayama et al., 2008; Wang et al., 2004; Zhang et al., 2010). FOXP1 immunoreactivity may be associated with the immunoreactivity of ERα and PgR in breast cancer, which may predict favorable prognosis in patients (Banham et al., 2005; Rayoo et al., 2009). A recent study showed that FOXP1 immunoreactivity was significantly enhanced in breast cancer samples for tamoxifen-treated patients without relapse, compared with samples for those with relapse within 5 years after surgery (Shigekawa et al., 2011). It was also demonstrated that a positive immunoreactivity for either FOXP1 or FOXA1 significantly correlated with better relapse-free and overall survivals for breast cancer patients with adjuvant tamoxifen therapy, compared with either FOXP1- or FOXA1-negative immunoreactivity (Ijichi et al., 2012). Univariate and multivariate proportional analyses showed that the relapse-free and overall survival rates were associated with FOXA1 and FOXP1 immunoreactivities. For the relapse-free survival, either FOXP1 or FOXA1 immunoreactivity was found to be a significant prognostic predictor through univariate analysis ($P=0.001$ and 0.002, respectively), whereas only FOXP1 immunoreactivity was a better prognostic predictor based on multivariate analysis ($P=0.026$). On the other hand, neither FOXP1 nor FOXA1 was significantly associated with overall survival by multivariate analysis. Notably, double-positive FOXP1 and FOXA1 immunoreactivities were significantly associated with more favorable prognosis for the relapse-free and overall survivals compared with either FOXP1- or FOXA1-negative immunoreactivity based on multivariate analyses ($P=0.002$ and 0.002, respectively). These findings suggest that the combined analyses of the FOXA1 and FOXP1 immunoreactivities provide powerful prognostic indicators for the patients with ERα-positive breast cancers treated with adjuvant tamoxifen therapy.

Carroll and his colleagues showed that FOXA1 also plays a role in the differential ER-binding events in the tumors with a poor outcome (Ross-Innes et al., 2012). Notably, an siRNA-mediated knockdown study showed that ERα signals, including ERα occupancy and estrogen-dependent cell growth, are FOXA1 dependent in both tamoxifen-sensitive and tamoxifen-refractory MCF-7 cells (Hurtado et al., 2011). Further studies are required to answer the question whether the ER/FOXA1-driven growth is associated with tumor recurrence in various stages of the disease.

4. CONCLUSIONS AND FUTURE DIRECTIONS

A recent genome-wide study using ChIP analysis with high-throughput sequencing revealed that FOXA1 is a critical transcription factor that contributes to most of the ERα-chromatin interactions and estrogen-dependent changes of gene expression (Hurtado et al., 2011). FOXA1 influences genome-wide chromatin accessibility of ERα in response to different ligands, including both estrogen and tamoxifen (Hurtado et al., 2011). Thus, FOXA1 is considered as a major determinant for estrogen–ERα activity and endocrine response in breast cancer cells. Since FOXP1 exhibits functions analogous to those of FOXA1 in the ER-mediated transcription and its immunoreactivity has a clinicopathological significance along with FOXA1 immunoreactivity in breast cancer, it is assumed that FOXP1 also plays an important role in the regulation of ERα activity and tamoxifen responsiveness in breast cancer, functioning cooperatively with FOXA1. Future genome-wide studies of FOXP1 binding as well as ERα and FOXA1 occupancy will elucidate the precise interactions of these transcription factors in the ERα-mediated signaling pathways.

In summary, *FOXP1* and *FOXA1* are primary ERα target genes and critical transcription factors that regulate the ERα activity. Both FOX proteins will be potential biomarkers for the prediction of breast cancer prognosis. Pharmacological modulation of FOXP1 and FOXA1 activities may be clinically useful in the prevention and/or treatment of breast cancer.

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PLoS one

Alterations of Gene Expression and Glutamate Clearance in Astrocytes Derived from an MeCP2-Null Mouse Model of Rett Syndrome

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Abstract

Rett syndrome (RTT) is a neurodevelopmetal disorder associated with mutations in the methyl-CpG–binding protein 2 (MeCP2) gene. MeCP2-deficient mice recapitulate the neurological degeneration observed in RTT patients. Recent studies indicated a role of not only neurons but also glial cells in neuronal dysfunction in RTT. We cultured astrocytes from MeCP2-null mouse brain and examined astroglial gene expression, growth rate, cytotoxic effects, and glutamate (Glu) clearance. Semi-quantitative RT-PCR analysis revealed that expression of astroglial marker genes, including GFAP and S100β, was significantly higher in MeCP2-null astrocytes than in control astrocytes. Loss of MeCP2 did not affect astroglial cell morphology, growth, or cytotoxic effects, but did alter Glu clearance in astrocytes. When high extracellular Glu was added to the astrocyte cultures and incubated, a time-dependent decrease of extracellular Glu concentration occurred due to Glu clearance by astrocytes. Although the shapes of the profiles of Glu concentration versus time for each strain of astrocytes were grossly similar, Glu concentration in the medium of MeCP2-null astrocytes were lower than those of control astrocytes at 12 and 18 h. In addition, MeCP2 deficiency impaired downregulation of excitatory amino acid transporter 1 and 2 (EAAT1/2) transcripts, but not induction of glutamine synthetase (GS) transcripts, upon high Glu exposure. In contrast, GS protein was significantly higher in MeCP2-null astrocytes than in control astrocytes. These findings suggest that MeCP2 affects astroglial genes expression in cultured astrocytes, and that abnormal Glu clearance in MeCP2-deficient astrocytes may influence the onset and progression of RTT.

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Introduction

Rett syndrome (RTT) is a neurodevelopmetal disorder that affects one in 15,000 female births, and represents a leading cause of mental retardation and autistic behavior in girls [1,2]. Mutations in the methyl-CpG–binding protein 2 (MeCP2) gene, located in Xq28, have been identified as the cause for the majority of clinical RTT cases [3]. Knockout mouse models with disrupted MeCP2 function mimic many key clinical features of RTT, including normal early postnatal life followed by developmental regression that results in motor impairment, irregular breathing, and early mortality [4,5,6]. MeCP2 dysfunction may thus disrupt the normal developmental or/and physiological program of gene expression, but it remains unclear how this might result in a predominantly neurological phenotype.

In several RTT mouse models, a conditional knockout that is specific to neural stem/progenitor cells or postmitotic neurons results in a phenotype that is similar to the ubiquitous knockout, suggesting that MeCP2 dysfunction in the brain and specifically in neurons underlies RTT [1,6,7]. Recent studies have demonstrated that mice born with RTT can be rescued by reactivation of neuronal MeCP2 expression, suggesting that the neuronal damage can be reversed [1,6]. In addition, several studies using in vitro cell culture systems also indicate that MeCP2 may play a role in processes of neuronal maturation including dendritic growth, synaptogenesis, and electrophysiological responses [1,7]. These data support the idea that MeCP2 deficiency in neurons is sufficient to cause an RTT-like phenotype. However, emerging evidence now indicates that MeCP2 deficiency in glia may also have a profound impact on brain function [8,9,10,11,12,13]. Brain magnetic resonance (MR) studies in MeCP2-deficient mice demonstrated that metabolism in both neurons and glia is affected [8]. Furthermore, in vitro co-culture studies have shown that MeCP2-deficient astroglia non-cell-autonomously affect neuronal dendritic growth [9,10]. In addition, MeCP2-deficient microglia cause dendritic and synaptic damage mediated by elevated glutamate (Glu) release [11]. Very recent studies have indicated that re-expression of MeCP2 in astrocytes of MeCP2-deficient mice significantly improves locomotion, anxiety levels, breathing patterns, and average lifespan, suggesting that astrocyte dysfunc-

tion may be involved in the neuropathology and characteristic phenotypic regression of RTT [13].

Astrocytes regulate the extracellular ion content of the central nervous systems (CNS); they also regulate neuron function, via production of cytokines, and synaptic function, by secreting neurotransmitters at synapses [14,15]. Moreover, a major function of astrocytes is efficient removal of Glu from the extracellular space, a process that is instrumental in maintaining normal interstitial levels of this neurotransmitter [16]. Glu is a major excitatory amino acid; excess Glu causes the degeneration of neurons and/or seizures observed in various CNS diseases [14,17]. RTT is also associated with abnormalities in Glu metabolism, but these findings are controversial due to the limitations of the experimental strategies used. Two studies have demonstrated that Glu is elevated in the cerebrospinal fluid (CSF) of RTT patients [18,19]. MR spectroscopy in RTT patients also revealed elevations of the Glu and Gln peak [20,21]. On the other hand, an animal MR study reported that the levels of Glu and Gln were decreased in a mouse model of RTT [8]. A more recent study indicated that MeCP2-null mice have reduced levels of Glu, but elevated levels of Gln, relative to their wild-type littermates [22]. Another study reported increased Gln levels and Gln/Glu ratios in Mecp2 mutant mice, but no decreases in Glu levels [23]. Although these in vivo studies have explored the hypothesis that the Glu metabolic systems might be altered in RTT, no solid conclusions have yet been reached [24,25].

In this study, we investigated the contribution of MeCP2 to the physiological function of astrocytes. Our studies demonstrate that MeCP2 is not essential for the growth and survival of astrocytes, but is involved in astrocytic Glu metabolism via the regulation of astroglial gene expression.

Results

Characterization of MeCP2-null astrocytes

It was recently reported that MeCP2 is normally present not only in neurons but also in glia, including astrocytes, oligodenrocytes, and microglia [9,10,11]. To determine the roles of MeCP2 in astrocytes, we cultured cerebral cortex astrocytes from both wild-type ($MeCP2^{+/y}$) and MeCP2-null ($MeCP2^{-/y}$) mouse brains (Fig. 1). MeCP2-null astrocytes exhibited a large, flattened, polygonal shape identical to that of the wild-type astrocytes, suggesting that normal patterns of cellular recognition and contact were present. Semi-quantitative RT-PCR using primer sets that specifically amplify two splice variants, Mecp2 e1 and e2, showed that control astrocytes expressed Mecp2 e1 and e2, whereas neither Mecp2 variant was detectable in MeCP2-null astrocytes (Fig. 1A). We further confirmed expression of MeCP2 by immunocytochemical staining of astrocytes. In control samples, almost all GFAP-positive cells exhibited clear nuclear MeCP2 immunoreactivity in astrocytes, but no immunoreactivity was observed in MeCP2-null astrocytes (Fig. 1B).

MeCP2 has been reported to be involved in regulation of astroglial gene expression [26,27]. Consistent with this, GFAP levels were significantly higher in MeCP2-null astrocytes (Fig. 1A). Similarly, the expression of S100β, another astrocyte maturation marker, was significantly upregulated by MeCP2 deficiency (fold change of control = 1.0, GFAP: 2.195 ± 0.504, $n=4$ each, $p<0.05$; S100β: 2.779 ± 0.329, $n=4$ each, $p<0.01$). These results show that MeCP2 deficiency upregulates astroglial gene expression in astrocytes.

To compare the growth of the wild-type and MeCP2-null astrocytes, we counted total cell number at each passage (Fig. 2A). As passage number increased, the cell growth rate decreased dramatically for both types of astrocytes, ultimately culminating in senescence. There was no significant difference in growth rate between the control and MeCP2-null astrocyte cultures. We then measured astrocyte proliferation via BrdU incorporation assay (Fig. 2B and Fig. S1). After 2 h of BrdU treatment, the proportions of BrdU-incorporating cells were similar in the control and MeCP2-null astrocytes ($6.635\pm1.655\%$ in control versus $6.774\pm2.272\%$ in MeCP2-null astrocytes, $n=4$ each, $p=0.962$). These results suggest that the absence of MeCP2 did not affect the proliferation of astrocytes in our culture condition.

We also tested the cytotoxic effects of hydrogen peroxide (H_2O_2), ammonium chloride (NH_4Cl), and glutamate (Glu), on astrocytes in our culture (Fig. 2C–E). In cultures derived from both wild-type and MeCP2-null strains, cell viability decreased with increasing concentrations of H_2O_2 and NH_4Cl. In contrast, in our culture conditions, we observed virtually 100% viability of both the control and MeCP2-null astrocytes after 24 h incubation with 10 mM Glu. Glu-induced gliotoxic effects have been previously reported by Chen et al. (2000), and are probably due to distinct differences in culture conditions, specifically the presence of glucose [28]. These results showed that H_2O_2 and NH_4Cl had a similar effect in both strains of astrocytes. There was no significant difference in viability between the control and MeCP2-null astrocyte cultures, indicating that MeCP2 deficiency did not affect astrocyte viability upon treatment with H_2O_2 and NH_4Cl.

Effects of glutamate on glutamate transporters and glutamine synthetase transcripts in MeCP2-null astrocytes

High extracellular Glu interferes with the expression of the astrocyte transporter subtypes, excitatory amino acid transporter 1(EAAT1)/glutamate/aspartate transporter (GLAST) and EAAT2/glutamate transporter-1 (GLT-1) [16,29]. To explore the effects of Glu on the expression of Glu transporter genes in cultured astrocytes from wild-type and MeCP2-null mouse brains, we asked whether treatment with 1.0 mM Glu altered expression of EAAT1 and EAAT2 mRNA, using a semi-quantitative RT-PCR assay (Fig. 3). EAAT1 and EAAT2 mRNA were expressed in both wild-type and MeCP2-null astrocytes, and were slightly higher in controls than in MeCP2-null astrocytes. Both EAAT1 and EAAT2 mRNA levels were altered in the control astrocytes after treatment with 1.0 mM Glu. EAAT1 mRNA levels decreased significantly in the wild-type astrocytes, both 12 h and 24 h after treatment with Glu (Fig. 3A). In contrast, EAAT1 decreased significantly in the MeCP2-null astrocytes, at 12 h but not 24 h after treatment. As with EAAT1, EAAT2 mRNA levels also decreased significantly in the control astrocytes, both 12 h and 24 h after treatment (Fig. 3B). However, EAAT2 decreased significantly in MeCP2-null astrocytes, 24 h but not 12 h after treatment. In addition, the effects of Glu on EAAT1 and EAAT2 relative fold expression at 12 h were altered in the MeCP2-null astrocytes (Fig. 3D: EAAT1; 0.618 ± 0.033 in control versus 0.758 ± 0.049 in MeCP2-null astrocytes, $n=10$ each, $p<0.05$; Fig. 3E: EAAT2; 0.794 ± 0.055 in control versus 0.964 ± 0.048 in MeCP2-null astrocytes, $n=8$ each, $p<0.05$). These results suggest that the loss of MeCP2 leads to transcriptional dysregulation of these genes, either directly or indirectly.

One important enzyme that plays a role in the Glu metabolic pathway is glutamine synthetase (GS) [17,29]. GS is mainly located in astrocytes; cultured astrocytes response to Glu with increased GS expression [17,29]. Consistent with this, 1.0 mM Glu treatment stimulated GS mRNA expression in both the wild-type and MeCP2-null astrocytes about 1.2-fold after 12 h but not 24 h (Fig. 3C). In addition, MeCP2 deficiency did not modify the