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STAT1 activation regulates proliferation and differentiation of renal progenitors

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Abstract

We have shown previously that activation of STAT1 contributes to the pathogenesis of Wilms tumor. This neoplasm caricatures metanephric development and is believed to originate from embryonic renal mesenchymal progenitors that lose their ability to undergo mesenchymal-epithelial transition (MET). Therefore, we hypothesized that STAT1 is also activated and functional during metanephric development. Here we have demonstrated that both STAT1 and STAT3 are activated during normal development of the embryonic kidney. Furthermore, activation of STAT1 stimulated the proliferation of metanephric mesenchymal cells, but it prevented MET and tubulogenesis induced by leukemia inhibitory factor, which preferentially activates STAT3. Consistent with its negative regulation of metanephric mesenchymal differentiation, inhibition of STAT1 activation with protein kinase CK2 inhibitor TBB or RNAi-mediated knockdown of STAT1 promoted differentiation of metanephric progenitors and abolished the effect of cytokine-induced STAT1 activation in these cells. Additionally, a cell-permeable peptide that inhibits STAT1-mediated transactivation by targeting the STAT1 N-domain also blocked cytokine-induced STAT1-dependent proliferation in metanephric progenitors and promoted LIF-induced MET and tubulogenesis. Finally, the STAT1 peptide inhibitor caused the down regulation of survival/anti-apoptotic factors, Mcl-1 and Hsp-27, and induced apoptosis in renal tumor cells with constitutively active STAT1, indicating that STAT1 is required for these cells to survive. These findings show that both metanephric progenitors and renal tumor cells utilize a STAT1-dependent mechanism for growth or survival.

Keywords

STAT1; STAT3; kidney; Wilms tumor; mesenchymal-epithelial transition; development

1. Introduction

The Signal Transducer and Activator of Transcription (STAT) proteins are intracellular mediators of the JAK/STAT signaling pathway, which is triggered by a large number of

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secreted growth factors and cytokines. STAT-like proteins have been identified in a variety of organisms, including *Drosophila*, *Xenopus*, and zebrafish and play fundamental roles in stem cell maintenance and migration [1-3]. In mammals, the STAT family of proteins is comprised of seven well-conserved members, which have been implicated in a wide variety of biological processes including cell growth, migration, differentiation, apoptosis, angiogenesis and oncogenesis [4-6]. Normally, STATs are transiently activated in response to extracellular signals, and this activation often occurs in a cell-cell context and specific developmental stage-dependent manner [7-10]. Upon cytokine binding to an appropriate receptor, a member of the Janus tyrosine kinase (Jak) family becomes activated, resulting in STAT phosphorylation, which in turn triggers STAT dimerization and nuclear localization. Once in the nucleus, the STAT oligomer binds to specific DNA response elements and promotes transcription [11,12]. STATs can also be phosphorylated by receptor tyrosine kinases and non-receptor tyrosine kinases such as Src and Abl, and by serine/threonine kinases such as MAPK, PKC, and protein kinase CK2 [13]. These modifications may regulate their transcriptional activity and are likely responsible for the crosstalk between different signal transduction pathways. Furthermore, phosphorylated as well as unphosphorylated STATs may function through direct interaction with cellular proteins such as glucocorticoid receptors, p300, and NF κ B [14,15].

Of the various family members, STAT1 and STAT3 have been identified as mediators of signaling induced by interferons and gp130 receptor-binding cytokines/IL-6 family members, respectively. They are not only critical signal transducers of native and acquired immunity and inflammation, but also important regulators of cell growth and oncogenesis [16]. In particular, the early embryonic lethality in *STAT3*-deficient mice indicates an essential role for this STAT in the growth and development of mesodermal tissues [17]. Furthermore, activated STAT3 is transforming and constitutively activated in a variety of hematological and solid human cancers [18]. These are characteristics of an oncogene which might be exploitable in cancer therapies [19]. On the other hand, loss of STAT1 enhances susceptibility to chemically induced cancers and broadens the tumor spectrum in p53-deficient mice, suggesting that it functions as a tumor suppressor under certain circumstances [20,21]. However, STAT1 is also activated in a variety of cancers [22], and ectopic expression of IFN- γ in the mouse central nervous system during development induces medulloblastomas that are STAT1-dependent [23], indicating that it can be oncogenic in some contexts.

During kidney development, renal mesenchymal progenitors that comprise the metanephric mesenchyme (MM) are induced to undergo mesenchymal-epithelial transition and differentiate into tubular epithelial cells, the major component of the nephron, by factors secreted from the ureteric bud [24-28]. One of these, leukemia inhibitory factor (LIF), activates STAT3 and induces mesenchymal progenitor cell differentiation [25,26,29]. Since Wilms tumor (WT), one of the most common childhood cancers, is thought to originate from aberrant or inhibited metanephric mesenchymal cell differentiation [30,31], we asked whether STATs play a role in its pathogenesis. Interestingly, STAT1, but not STAT3, is constitutively activated in most Wilms tumors and contributes to the survival and growth of Wilms tumor cells by upregulation of genes directly linked to tumorigenesis, namely *Mcl-1* and *Hsp27* [32]. This led us to examine whether STAT1 might also be involved in regulating the proliferation and differentiation of renal mesenchymal progenitor cells. Here we report that, in addition to STAT3, STAT1 is also activated during metanephric development. In isolated metanephric mesenchymes, activation of STAT1 promotes the proliferation of mesenchymal progenitors and inhibits LIF-induced MET and tubulogenesis, whereas inhibition of STAT1 promotes the differentiation of mesenchymal cells. A specific peptide inhibitor of STAT1 also induces apoptosis in renal tumor cell lines with activated STAT1. These results indicate that STAT1 plays an important role in regulating the

proliferation and differentiation of metanephric mesenchymal cells. It also appears that metanephric mesenchyme and Wilms tumors may share a STAT1-dependent survival mechanism that could be targeted for therapeutic purposes.

2. Materials and Methods

2.1. Reagents

These included recombinant rat interferon gamma (IFN- γ) (BD Pharmingen (San Diego, CA) and TBB (Calbiochem, San Diego, CA). The antibodies included anti-pY701 STAT1, anti-pY705 STAT3, anti-STAT3 (Cell Signaling Technology, Danvers, MA); mouse anti-pS727 STAT1 antibody and anti-pS10 Histone H3 antibody (Upstate Biotechnology (Millipore), Lake Placid, NY); rabbit anti- α -Actin antibody (Sigma, St. Louis, MO); Anti-MCL-1, rabbit anti-STAT1 and anti-PARP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA,); Anti-HSP27 and anti-HSP70 antibodies (Abcam Inc., Cambridge, MA); Rhodamine-, Alex-488-, or HRP-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Annexin-V-PE apoptosis detection kit was from BD Biosciences, San Diego, CA. Peptide inhibitors were synthesized as previously described [33].

2.2. Cell lines and cell culture conditions

Human embryonic kidney (HEK) 293 cells and human kidney tumor cell line SK-NEP-1 were from ATCC (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, 50/50; Gibco-BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, 100 μ g/ml streptomycin (Cellgro, Mediatech, Herndon, VA), and 2 mM glutamine in a humidified 5% CO₂ incubator. The conditionally immortalized RIMM-18 (Rat Inducible Metanephric Mesenchyme) [29] and U3A (STAT1 deficient) [33] lines were cultured as described previously.

2.3. Cell proliferation assay

Cells ($2-4 \times 10^3$ cells per well) were plated in a 96-well plate and treated as indicated for 24-72 hours. Cell viability and growth were assessed by the WST-1 cell proliferation reagent (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions.

2.4. Annexin V staining

Annexin V-PE staining was performed according to manufacturer's instruction. 1×10^6 cells per well were plated in 6-well plate and treated with or without H2-2 in DMEM/F12 medium supplemented with 0.5 % FBS for 2 hours. FACS analysis was used to quantitatively determine the percentage of Annexin V-PE positive, 7AAD negative cells that are actively undergoing apoptosis.

2.5. Animals and Metanephric Mesenchymes

All animal procedures were performed following the guidelines from the NCI-Frederick Animal Care and Use Committee. *Stat1*^{-/-} tissues were generated in collaboration with Giorgio Trinchieri from mice kindly provided by David E. Levy [34]. Metanephric mesenchymes dissected away from T-shaped ureteric buds were prepared from E13.5 rat embryos and cultured as described previously [35]. Cultured mesenchymes were treated with a 50x stock solution of CM from a rat ureteric bud cell line (CM) or LIF to induce epithelial differentiation, as described previously [27] or with other reagents as needed (i.e., 30-50 ng/ml human FGF2 (R&D), 5-30 ng/ml recombinant human LIF (Sigma), 10 ng/ml human TGF- β (Sigma)).

2.6. Immunoblot analysis

After treatments, cells were washed and scraped with PBS, pH 7.4, pelleted, and resuspended in ice-cold cell lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L EDTA) containing protease inhibitors (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails (Sigma). Cell extracts were incubated for 15 min on ice, recovered by centrifugation at 14,000 x g for 15 min, and either used immediately or stored at -80°C. Extracts (50 µg of total protein) were boiled in Laemmli reducing sample buffer and resolved in 10% NuPAGE gels (Invitrogen) for 2-4 hours at 60 V. Proteins were electrotransferred to PVDF membranes (Bio-Rad, Hercules, CA), and membranes were blocked with 5% nonfat dry milk or 5% BSA in TBST, and incubated overnight at 4°C with primary antibodies according to recommended protocols. Membranes were washed with TBST, followed with species-specific secondary antibodies (1/10,000), and bound antibodies were visualized using the SuperSignal West Pico Chemiluminescent Substrate system according to the instructions of the manufacturer (Pierce, Piscataway, NJ). All experiments were conducted independently at least three times.

2.7. RNA interference

Specific short hairpin RNA (shRNA) against STAT1 or scrambled shRNA plasmids were obtained from SABiosciences (SABiosciences, Frederick, MD, USA). RIMM cells or MMs were transfected by the Amaxa Nucleofector® 96-well Shuttle™ System (Lonza Group Ltd.). Nucleofections were performed according to the manufacturer's instructions using program CC112. Briefly, $0.5-1 \times 10^5$ cells per transfection were resuspended in 20 µl 96-well Nucleofector Solution. 0.4-1 µg plasmid DNA was mixed with the cell suspension and transferred to the well of a 96-well Nucleocuvette® module. Protein lysates from cultured cells were prepared 24-48h after transfection. RNAs were extracted from treated tissues 3 or 6 days after transfection and induction.

2.8. Semi-quantitative RT-PCR

RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA), and RNase-Free DNase Set (Qiagen, Valencia, CA) was used to digest DNA during RNA purification. Reverse transcription (RT) reactions and Polymerase chain reaction (PCR) were performed as previously described. Primer sequences, annealing temperatures, and cycle numbers were previously described [29]. No RT controls were performed to rule out DNA contamination.

2.9. Whole mount (WISH) and frozen section *in situ* hybridization

WISH was performed essentially as described previously [36], except that BM purple (Roche) was used as the chromogenic substrate. Prior to the *in situ* hybridization procedure, the tissues or explants were treated as previously described [37]. Frozen sections for *in situ* hybridization were prepared and processed as previously described [38].

2.10. Immunocytochemistry

For immunocytochemistry (ICC), metanephric mesenchymes were cultured and treated as indicated. Then the cells were fixed with ice-cold methanol for 10 min, and labeled with 1:50 anti-pS10 Histone H3 antibody and 1:100 Alexa 488 donkey anti-rabbit secondary antibody; or fixed, stained, and visualized with Vimentin (monoclonal antibody, 1:50) or E-cadherin (polyclonal antibody, 1:50) (Santa Cruz Biotechnology) using rhodamine- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100) (Santa Cruz Biotechnology) as described previously [29]. Vimentin and E-cadherin antibodies labeled a single band of appropriate mass in immunoblots (Figure S1), demonstrating their specificity in these studies. Images were captured with a Zeiss Axiovert 200M fluorescent microscope.

2.11. Statistical analysis

Data were evaluated for statistical significance by analysis using student t-test. Statistical difference was considered to be significant if $p < 0.05$ (*) or $p < 0.01$ (**). All experiments were performed independently at least three times, and the data presented are from a representative experiment. The results are presented as mean $\pm$ S. D. (error bars).

3. Results

3.1. STAT1 is activated in developing kidney and metanephric mesenchymal progenitors

The constitutive activation of STAT1 in Wilms tumors prompted us to investigate whether STAT1 is also activated during normal development of the kidney. Adult and developing rat kidneys at E13.5 (when the metanephros is just forming) to E19.5 were dissected and lysed for immunoblotting with anti-phospho-STAT1 and STAT3 antibodies. Consistent with the established role of LIF in the differentiation of metanephric mesenchyme [25,26], STAT3 is phosphorylated on Y705 in early developing kidney, starting from E13.5, when the metanephric rudiment first appears. Interestingly, both tyrosine (p-Y701) and serine (p-S727) phosphorylated forms of STAT1 were detected in the developing kidney (Figure 1A) beginning at E13.5, indicating that STAT1 is also activated in early renal development. Although both STAT3 and STAT1 are present in adult rat kidney, little phosphorylated STAT1 and STAT3 can be detected, indicating that they are not activated once development ceases (Figure 1A). To further understand the potential role of STAT1 activation in kidney development, use was made of isolated embryonic rat MMs, which undergo MET and tubulogenesis in response to LIF in an explant culture system [25,26]. Treatment of the metanephric mesenchymes with LIF induced phosphorylation of STAT3 at Y705 as well as phosphorylation of STAT1 at Y701 and S727 (Figure 1B), whereas IFN- γ induced STAT1, but not STAT3, phosphorylation in these cells (Figure 1C), indicating that IFN- γ can be used as a tool to assess the role of activated STAT1 in kidney development. That is not to suggest, however, that it is the endogenous ligand responsible for STAT1 activation in the metanephros.

3.2. IFN- γ promotes proliferation of metanephric mesenchymal progenitors and represses their differentiation

We first asked whether IFN- γ could affect the proliferation or differentiation of mesenchymal cells in our explants culture system. Unlike LIF (Figure 2A, right panel), IFN- γ does not induce MET or tubulogenesis (Figure 2A, middle panel) in cultured mesenchymes assessed both by morphology and by expression of *Lim-1*, a differentiation marker that is up-regulated in newly formed epithelial tubules in whole mount *in situ* hybridization (WISH) assays. However, mesenchymes that were treated with IFN- γ and cultured for 6 days (Figure 2A, middle panel) were significantly larger than the controls (Figure 2A, left panel). Immunostaining with an antibody to phospho-Histone H3, a mitotic marker, indicated that there are significantly increased numbers of mitotic cells in IFN- γ -treated mesenchymes (Figure 2B). IFN- γ -induced cell proliferation became evident after one day of treatment, as measured using the mitochondrial dehydrogenase substrate WST-1, and reached a plateau after 3 days (Figure 2C). Thus, IFN- γ stimulates proliferation of mesenchymal cells but does not induce their differentiation.

As shown in Figure 3A, LIF or rat ureteric bud cell conditioned medium (CM) [27] induced the expression of *Lim-1* and differentiation of mesenchymal cells as revealed by WISH (a, c) and H&E staining (e). The presence of IFN- γ with LIF or CM suppressed *Lim-1* induction (b, d) and tubulogenesis (f). The suppression of LIF-induced differentiation markers *Lim-1* and *E-cadherin* by IFN- γ was also demonstrated by semi-quantitative RT-PCR analysis (Figure 3B). As controls, cell condensation markers *Pax-2* and *Pax-8* were not significantly

affected by IFN- γ (Figure 3B). To validate the RT-PCR results and to demonstrate that tubular markers are affected at the protein level, we also evaluated expression of Lim-1 and E-cadherin by immunoblotting. Expression of both markers was induced with LIF treatment and this effect was abrogated by the simultaneous addition of IFN- γ (Figure 3C), which is consistent with our findings from RT-PCR analyses. Furthermore, immunostaining with antibodies against vimentin, a mesenchymal cell marker, and E-cadherin, an epithelial marker showed that IFN- γ -exposed mesenchymes were vimentin-positive and E-cadherin-negative (Figure 3D, upper panels), whereas LIF treatment resulted in the reduction of vimentin expression and induction of E-cadherin (middle panels). Co-treatment of cultures with IFN- γ and LIF preserved vimentin expression and prevented the induction of E-cadherin (lower panels). Taken together, these results indicate that IFN- γ stimulates proliferation of mesenchymal cells and abrogates LIF-induced MET and tubulogenesis.

3.3. Inhibition of STAT1 activation promotes mesenchymal cell differentiation

The effects of IFN- γ on MET and tubulogenesis indicated that activation of STAT1 might play an important role in the proliferation and differentiation of mesenchymal cells. We have previously demonstrated that casein kinase II (CKII) regulates STAT1 activation in Wilms tumor cells, and CKII inhibitor TBB (4, 5, 6, 7-tetrabromo-2-benzotriazole) prevents the activation of STAT1 [32]. As shown in Figure 4A, the presence of TBB significantly enhanced the induction of *Lim-1* in cultured mesenchymes. RT-PCR analysis showed that the presence of TBB not only accelerated expression of *E-cadherin* and *Lim-1* in metanephric mesenchymes as early as 3 days after LIF treatment (data not shown), but also largely abolished IFN- γ -mediated inhibition of *E-cadherin* and *Lim-1* expression (Figure 4B). H&E staining of tissue sections confirmed that the presence of TBB overcame the inhibitory effect of IFN- γ on LIF-induced tubulogenesis (Figure 4C).

To further address the role of STAT1 in the differentiation of metanephric mesenchyme, a plasmid expressing the short hairpin RNA (shRNA) targeting STAT1 was used to down-regulate STAT1. A plasmid that expresses a scrambled RNA was used as a control in all the experiments. Transfection of the STAT1-targeting plasmids into RIMM-18 cells, a line derived from metanephric mesenchymal progenitors (Levashova, et. al., 2003), reduced both STAT1 mRNA and protein without affecting the level of STAT3 (Supplemental Figure S2). In mesenchymes transfected with the plasmid expressing STAT1-specific shRNA, the level of STAT1 mRNA was decreased (Figure 4D). Moreover, it prevented the inhibition mediated by IFN- γ on LIF-induced expression of E-cadherin and Lim-1 (Figure 4E). The effects of knockdown of STAT1 on the expression of vimentin and E-cadherin in metanephric mesenchymes were further examined by immunostaining (Figure 4F). While IFN- γ inhibited the differentiation of mesenchymal cells and sustained the expression of vimentin, knockdown of STAT1 significantly reduced the expression of vimentin. It also enhanced expression of the epithelial marker E-cadherin (Figure 4F). These data demonstrate that STAT1 mediates the inhibitory effect of IFN- γ on the differentiation of metanephric mesenchyme.

3.4 Generation and characterization of specific STAT1 inhibitors

Since we had previously reported the efficacy of targeting STAT3 using a cell-permeable STAT3-specific peptide inhibitor [39], we employed a similar approach in generating a STAT1-specific peptide inhibitor to use as a chemical biology tool to study the roles of STAT1. Dimerization through its N-domain is essential for STAT1 transcriptional activity [40]. However N-domain interactions are not conserved in the STAT family, because the N-domain dissociation constants of STAT1, STAT3, and STAT4 differed by more than three orders of magnitude. Based on this fact, we hypothesized that inhibition of STAT1 N-domain dimerization may allow for the selective inhibition of STAT1. The crystal structure

of STAT1 revealed that the second helix of the transcription factor is an essential part of the dimer interface [41]. We therefore prepared peptides corresponding to the second helix of STAT1 and fused them to the cell-penetrating peptide penetratin for easier intracellular delivery. We used the reported STAT1 tertiary structure to optimize the structure of inhibitors applying the strategy that was used previously in the development of STAT3 inhibitors (33). In the tertiary structure of non-phosphorylated STAT1, Gln 20 appears to form a hydrogen bond with Asp575 of the linker domain [41]. We assumed that substitution of the hydrogen bond with a stronger ion pair interaction would enhance peptide binding to STAT1. Indeed, replacement of neutral Gln20 with positively charged Lys resulted in peptide H2-2 which proved to be the most potent among the penetratin-conjugated peptides. It was used to block STAT1 activity in all subsequent experiments. The H2-2 sequence was used for generating a fluorescent helix-2 analog for studying interactions with STAT1. We synthesized the H2-2 derivative with an additional Cys residue positioned at the C-terminus of penetratin: LDSKFLEQVHKLYRQIKIWFQNRRMKWKKC. Cys was used for labeling of the peptide with the maleimide derivative of tetramethyl rhodamine (TMR) to obtain TMRH2A-2 peptide. To determine whether TMR-H2-2 interacts with STAT1 in living cells, we used Fluorescence Resonance Energy Transfer (FRET) that allows detecting short-range (less than 10 nm) interactions in live cells. Briefly, HEK293 cells, stably transfected with vector expressing STAT1 tagged with eGFP on its N-terminus (eGFP-STAT1), were treated for one hour with 5 μ M TMR-H2-2. The image analysis was performed as described [39]. The photobleaching of TMR in a selected region restored the fluorescence of eGFP, suggesting that TMRH2-2 interacts with e-GFP-STAT1, but not e-GFP (Figure 3B). FRET efficiency (EF) was very high ($88.89 \pm 3.34\%$), suggesting tight binding of TMR-H2-2 to e-GFP-STAT1 (Supplementary Figure S3).

To demonstrate the specificity of H2-2 on STAT function, we also assessed its effects using STAT reporter constructs. In these studies, H2-2 inhibits the IFN- γ -induced increase of luciferase activity in RIMM cells transfected with the ISRE (Interferon-Stimulated Responsive Element) luciferase reporter (Supplemental Figure 3A). In contrast, H2-2 did not affect LIF-induced transactivation of the APRE (Acute-Phase Response Element) luciferase reporter activity, which is dependent upon STAT3 activation (Supplemental Figure 3B). Conversely, ST3-Hel2A-2, a peptide that interacts preferentially with the N-domain of STAT3, specifically inhibited the STAT3-dependent APRE reporter but not the IFN- γ -dependent ISRE reporter. These results suggest that H2-2 is a cell-permeable inhibitor that selectively blocks STAT1 activity. In the case of STAT4, analogs of the second helix distorted the structure and assembly of almost the entire N-domain, as revealed by NMR studies [33]. We speculate that a similar mechanism may be responsible for the inhibitory effects of the second helix analogs of STAT1.

3.5 A STAT1-specific peptide inhibitor H2-2 promotes differentiation in metanephric mesenchymes

The constitutive activation of STAT1 in Wilms tumors and the impact of STAT1 on mesenchymal cell proliferation and differentiation suggested that this transcription factor may serve as a significant target for therapeutic targeting in renal tumor cells.

To assess its effects on mesenchymal cells, isolated tissues were exposed to H2-2 for 3 or 6 days. Compared with mesenchymes treated with LIF alone, the presence of the H2-2 inhibitor accelerated and enhanced the induction of differentiation markers *Lim-1* and *E-cadherin* (Figure 5A, upper panel). H&E staining of a tissue section showed that the inhibitor-treated tissues had greatly increased tubule formation (Figure 5A lower panel). Furthermore, H2-2 treatment significantly reduced the number of mitotic mesenchymal cells stimulated by IFN- γ (Figure 5B). These data demonstrate that H2-2 is an effective specific

inhibitor of STAT1 and provide additional evidence for the regulatory role of STAT1 in mesenchymal cell growth and differentiation.

3.6 Inhibition of STAT1 induces growth arrest in kidney tumor cells with activated STAT1

Since our previous studies found that constitutive activation of STAT1 contributes to the pathogenesis of Wilms tumor [32], we asked whether the STAT1 inhibitor H2-2 could affect the growth and survival of kidney-derived transformed cell lines HEK293 and SK-NEP-1, which have constitutively activated STAT1 [32]. As shown in Figure 5C, 5-10 μ M of H2-2 effectively suppressed the growth of tumor cells, but not that of STAT1 deficient U3A cells or RIMM-18 cells, which do not have constitutively activated STAT1. At least part of the growth suppression is due to increased levels of apoptosis as demonstrated by the elevated percentage of Annexin V-positive cells in H2-2-treated tumor cells (Figure 5D). Consistent with our previous findings that MCL-1, HSP27 and HSP70 are downstream mediators of STAT1 signaling [32], H2-2 decreased the levels of MCL-1, HSP27, and HSP70 in SK-NEP-1 cells but not in STAT1-deficient U3A cells (Figure 5E). These results indicate that H2-2 specifically acts on STAT1 and induces growth arrest and apoptosis in transformed cells possessing active STAT1.

4. Discussion

A cell's decision to continue to proliferate as a progenitor or to commit to differentiation and subsequently undergo apoptosis is fundamental to the survival of an organism. It is a tightly controlled process within any given tissue with checks and balances that prevent the extremes of unregulated growth or cell death that are characteristic of pathologic conditions. In the current study, we have found that STAT1 is activated in the developing kidney. In cultured metanephric mesenchymes, activation of STAT1 with IFN- γ promotes the proliferation of mesenchymal cells and inhibits their differentiation, whereas inhibition of STAT1 accelerates the differentiation of these mesenchymal cells. These results indicate that STAT1 can function as a potent regulator of metanephric development. Similarly, IFN- γ or activation of STAT1 is also implicated in the development of the cerebellum, mammary gland and lens development [7,42-44]. Interestingly, it has been found that the loss of STAT1 inhibitor SOCS1 impairs osteoblast differentiation [45], raising the possibility that STAT1 functions as a general regulator of embryonic progenitors.

While most mouse genetic studies to date implicate STAT1 signaling in immune responses to pathogens, its role during development has been largely ignored. This is due in part to the complex nature of compensatory responses that have now been discerned for STAT family members, i.e., the numerous signaling ligands and their ability to activate multiple STAT family members, the induction of signaling through alternative STATs following a STAT deletion, and the modifying role of STAT inhibitors such as the SOCS family members, which are also downstream targets of STAT signaling. Our own review of tissues from *Stat1* KO mice has revealed no apparent phenotype in the developing metanephros (unpublished observation). This could be explained by such adaptive mechanisms, since our studies indicate that survival, growth and development normally depend, at least in part, upon STAT1 activity. For example, loss of SOCS3 leads to increased phosphorylation of STAT3 as well as STAT1, producing an IFN-like gene expression profile along with expression of STAT3 targets [46]. Furthermore, IFN- $\bullet$ signaling in STAT1-deficient cells results in upregulation of target genes for other STAT family members [47]. Such responses may therefore help to compensate for the loss of a specific STAT signal. Ultimately, the most informative and effective approach to address this possibility in the context of our models would be to study the loss and gain of function of *Stat1* in a tissue-specific manner, and these studies are ongoing.

The actual ligand responsible for STAT1 activation in the developing kidney is unclear. There are several candidates that may contribute; however, it is unlikely to be IFN- $\bullet$. Indeed, we have analyzed RNA samples from normal embryonic kidneys (from E13.5 to E19.5) and could barely detect mRNA expression for IFN- γ by RT-PCR (Wang and Perantoni, unpublished observation). It is conceivable that it is instead one of the several gp130-binding proteins (IL-6 family members), which have been identified in the developing kidney such as LIF [25,26], oncostatin-M [48], cardiotrophin [49], or ciliary neurotrophic factor [26], assuming they behave in the metanephros to activate STAT1 as has been reported in other tissues. Alternatively, activation may occur through cross-talk with a different pathway. For example, FGF2 and FGF8b can activate STAT1 but not STAT3 in vascular endothelial cells [50]. Such a mechanism may also be operative in metanephric mesenchyme, since Fgf8 is essential for nephron formation and its loss results in apoptosis of renal progenitors [37]. This possibility therefore warrants further investigation.

LIF is secreted by the inductive ureteric bud and induces the differentiation of mesenchymal cells during kidney development [25,26]. As a member of the IL-6 cytokine family that signals through gp130-containing receptors, LIF activates both STAT1 and STAT3 in mesenchymal cells (Fig. 1B). Our findings that activation of STAT1 prevents LIF-induced mesenchymal cell differentiation and that inhibition of STAT1 promotes their differentiation suggest that STAT3 mediates MET and tubulogenesis induced by LIF in this tissue. Consistent with this observation, it has also been shown that STAT3 is required for hepatocyte growth factor-induced MET and tubulogenesis during liver development [51]. Therefore, it is conceivable that activation of STAT1 by LIF modulates or limits the pro-differentiation effects of STAT3 in these cells. Alternatively, the presence of phosphorylated STAT3 may promote the formation of STAT1/STAT3 heterodimers that transactivate the genes for mesenchymal cell differentiation.

There have been extensive studies of the molecular alterations that lead to Wilms tumor. Mutations in WT1, WTX, and β -catenin may account for 30-35% of sporadic cases [52]. For the majority of Wilms tumors [53]/nephroblastoma [54], however, the critical molecular event(s) that leads to tumorigenesis remains unknown. Based on the pathological characteristics of Wilms tumors, it is believed that renal progenitors fail to differentiate and continue to proliferate as an important initial step in the disease process. Our findings suggest that the abnormal activation of STAT1 may abrogate the process of normal tubule formation from metanephric mesenchyme, resulting in the accumulation of blastemal/mesenchymal cells. This is consistent with our previous observation that STAT1 is constitutively activated in Wilms tumors [32]. Furthermore, it has been shown that IFN- $\bullet$ is produced by adrenal gland and kidney tumors, suggesting it may play a role in the development of these neoplasms [55]. Studies have also shown that overexpression of IFN- γ in neurons can induce medulloblastomas [23]; moreover, the cerebellum is sensitive to IFN- $\bullet$ -induced medulloblastoma only during a very narrow developmental stage [23]. Once the tumors are established, they become independent of continued IFN- $\bullet$ expression, further suggesting that the biological functions of STATs depend on the timing, magnitude, duration, and balance of STAT activation. We also noted that IFN- $\bullet$ treatment can reverse MET/differentiation in metanephric mesenchymes only during the inductive phase of differentiation (Wang et al, unpublished observation). The establishment of a transgenic model with tissue-specific and/or timed expression of IFN- $\bullet$ will be useful to study the underlying mechanisms. It would be interesting to evaluate whether enforced expression of IFN- γ or STAT1 in metanephric mesenchymes at certain stages can alter differentiation and induce nephroblastomas.

The oncogenic role of STAT3 in many tumors has made it an attractive target for developing new cancer therapeutics [56,57]. There have been a number of inhibitors that target STAT3

SH2 domain or upstream kinases that phosphorylate STAT3 [57]. The N-domain of STAT3 is the most diversified region among STAT family proteins and is required for their transactivation activities, presumably due to its involvement in oligomerization and in interactions with proteins of the transcription machinery and chromatin [58,59]. We previously reported that targeting the N-domain of STAT3 with cell-permeable analogs of the second helix specifically inhibits STAT3-dependent transactivation and preferentially induces growth arrest and apoptosis in a number of tumor cells [39]. A similar approach was used to design a cell-permeable peptidic inhibitor for STAT1. The results presented here indicate that H2-2 is a STAT1-specific inhibitor that can cause growth arrest and apoptosis in transformed cells harboring constitutively activated STAT1. Therefore, binding to the N-domain appears to be an effective and specific means to target STAT1. These peptidic inhibitors may be promising leads for new cancer therapeutic approaches.

In summary, our work provides a better understanding of the physiological role of STAT1 in regulating the differentiation of metanephric mesenchymal progenitor cells and of the potential connection between STAT1 signaling and abnormalities of early kidney development, such as Wilms tumor. Defining the down-stream targets of STAT1 and how STAT1 and STAT3 cooperate to regulate the differentiation of mesenchymal cells may provide important clues for more effective targeting of Wilms tumor and other cancers with aberrant STAT1 activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. STAT1 and STAT3 are activated in the developing kidney and differentially activated by LIF and IFN- γ

A. Adult and E13.5, E14.5, E15.5, E16.5, E19.5 embryonic rat kidneys were dissected and snap-frozen. Tissues were lysed in ice-cold cell lysis buffer as described in M&Ms. Equal amounts of proteins were separated in 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with antibodies against STAT1, p-Y701 STAT1, p-S727 STAT1, STAT3, p-Y705 STAT3, or β -Actin. Specific bands were visualized with HRP-conjugated secondary antibodies and enhanced chemiluminescence. B + C. LIF activates STAT1 and STAT3 in MMs, but IFN- γ activates only STAT1 in metanephric mesenchymes. Tissues were treated with LIF (30 ng/ml) or IFN- γ (30 ng/ml) for 15 mins. Tissues were harvested and immunoblotted with antibodies against STAT1, p-Y701 STAT1, p-S727 STAT1,

STAT3, p-Y705 STAT3, or β -Actin. C.LIF (30 ng/ml) activates STAT3 and STAT1 (p-Y701 & p-S727) in mesenchymes. D. IFN- γ (30 ng/ml) activates STAT1 (p-Y701 & p-S727) but not STAT3 in mesenchymes.

Figure 2. IFN- γ treatment stimulates proliferation of metanephric mesenchymes

A-C. IFN- γ alone stimulates proliferation of metanephric mesenchymes. Tissues were treated with IFN- γ (100ng/ml) for 6 days. A. Tubules were detected by Whole mount in situ hybridization (WISH) using a *Lim-1* anti-sense RNA Probe. Tissues treated with IFN- γ (middle panel) do not express differentiation marker *Lim-1* (positive control, LIF treatment, right panel) but have enlarged relative to control (PBS) treatment (left panel) and have increased numbers of cells labeled with mitotic marker p-Histone H3 (green) (B: a, b), DAPI (blue); Fluorescent cells were counted in five randomly chosen views of images. The graph represents the mean $\pm$ s.d. (n=5) (Figure 2B: c). C. MM cells were treated with IFN- γ or PBS in 96-well plates and subjected to WST-1 assay at the different time points indicated.

Figure 3. IFN- γ treatment represses MET/differentiation of metanephric mesenchymes

A. IFN- γ treatment blocks differentiation of metanephric mesenchymes induced by CM (A: a, b) or LIF (A: c, d). Mesenchymes were treated with CM (LIF) with or without IFN- γ pre-treatment for 6 days. Tubules were detected by Whole mount in situ hybridization (WISH) using a *Lim-1* anti-sense RNA Probe. H&E staining of sectioned tissue samples confirmed that tubules (white arrows) induced by LIF (A: e) were inhibited by IFN- γ pre-treatment (A: f). B. Mesenchymal Epithelial Transition (MET) was detected by expression of differentiation and epithelial markers *Lim-1* and *E-cadherin* or condensation markers *Pax-2* and *Pax-8* as assessed by RT-PCR. Mesenchymes were treated with LIF in combination with or without IFN- γ (0, 10, 30, 100 ng/ml) for 6 days. C. Mesenchymes were treated with LIF with or without IFN- γ (100 ng/ml) and evaluated by immunoblot for expression of tubular epithelial markers *Lim-1* and *E-cadherin*. The expression of both differentiation markers was attenuated by IFN- γ treatment. D. Mesenchymes were treated with IFN- γ , LIF, or LIF in combination with IFN- γ . 6 days after treatment, tissues were fixed with ice-cold methanol and immunocytochemical staining with antibodies against Vimentin (red) or E-cadherin (green); Nuclei were stained with DAPI (Blue). IFN- γ itself does not induce MET/differentiation in MMs as indicated by strong Vimentin expression with little expression of epithelial marker E-cadherin (C, upper panels). LIF induces MET/differentiation as indicated by a marked increase of E-cadherin expression (C, middle panels), but the expression of E-cadherin was abrogated by IFN- γ pre-treatment (C, lower panels).

Figure 4. Blockage of STAT1 function promotes differentiation and restores tubule formation of metanephric mesenchymes abrogated by IFN- γ

A. TBB treatment enhances tubule formation of mesenchymes (0, 5 μ M). Tissues were treated with LIF with/without TBB as indicated above for 6 days. Tubules were detected by WISH using a *Lim-1* anti-sense RNA Probe. B-C. TBB treatment restores tubule formation in mesenchymes inhibited by IFN- γ . Isolated tissues were treated as indicated and then used to isolate RNA after 6 days of induction. B. Semi-quantitative RT-PCR was used to detect expression of tubule epithelial markers *Lim-1* and *E-cadherin*. *Gapdh* was used for the cDNA loading control. LIF induces tubule formation and expression of *Lim-1* and *E-cadherin*, and TBB treatment restores tubule formation and up-regulates expression of *Lim-1*

and *E-cadherin* inhibited by IFN- γ . C. H&E staining confirmed the inhibitory effect of IFN- γ , and TBB treatment restores tubule formation. D-F. Knock down of STAT1 promotes tubule formation in mesenchymes. D. STAT1 was knocked down effectively and specifically by STAT1 shRNA. Plasmid expressing scrambled shRNA vector or the plasmid expressing STAT1-targeted shRNA were transfected into mesenchymes, and cells were harvested for RT-PCR analysis 48 hours after transfection. E. Mesenchymal cells were transfected with scrambled control or shRNA-STAT1 expressing vectors and tissue differentiation was measured by tubular epithelial markers *Lim-1* and *E-cadherin* after 6 days induction by LIF with or without IFN- γ pretreatment. F. Knock down of STAT1 rescues inhibition effect by IFN- γ pre-treatment. IFN- γ inhibits MET/differentiation in mesenchymes induced by LIF as indicated by strong Vimentin expression with little expression of epithelial marker E-cadherin (F, upper panels), but knock down of STAT1 restored the expression of E-cadherin abrogated by IFN- γ pre-treatment (F, lower panels).

Figure 5. STAT1 inhibitor H2-2 treatment accelerates MMs differentiation and inhibits cell proliferation and survival in Wilms tumor cells

A. STAT1 inhibitor H2-2 treatment accelerates differentiation in metanephric mesenchyme. Tissues were treated with or without H2-2 at different time points as indicated and subjected to Semi-quantitative RT-PCR for expression of either *Lim-1* or *E-cadherin*. H&E staining of sectioned tissue samples confirmed that 10 μ M H2-2 treatment (right panel) induces massive tubule formation compared with DMSO control treatment (left panel). B. IFN- γ -stimulated proliferation in mesenchymes is STAT1-dependent. Tissues were pre-treated with or without H2-2 for 30 mins and then exposed to IFN- γ for 24 hours. Mesenchymes treated with H2-2 and IFN- γ have significantly fewer cells expressing mitotic marker p-Histone H3 (green), DAPI (blue). Mitotic cells were counted in five randomly chosen fields. The graph represents the mean $\pm$ S.D. (n=5).

C-E. H2-2 inhibits proliferation and induces apoptosis in kidney tumor cells with activated STAT1. C. Inhibition of STAT1 attenuates growth and induces death of kidney cancer SK-NEP-1, WiT49, and HEK293 cells, but not normal kidney RIMM cells or STAT1-deficient cells U3A. Cells were plated a day before treatment. Growth inhibition was assayed by MTT analysis after 48h H2-2 treatment. D-E. Inhibition of STAT1 induces apoptosis of kidney cancer cells, but not normal cells. D. Normal kidney cells RIMM-18, STAT1 deficient cells U3A, kidney cancer cells SK-NEP-1, WiT49, and HEK293 cells were treated with 5 μ M H2-2 or DMSO as control for 2 hours. Early apoptosis was evaluated by an AnnexinV-PE staining kit and flow cytometry analysis after treatments. E. H2-2 treatment induces apoptosis by targeting heat shock proteins in cancer cells. SK-NEP-1 cells and U3A cells were treated with 5 μ M H2-2 at the time indicated. Western blot analysis was performed with antibody against MCL-1, HSP-27, HSP70 or β -Actin.