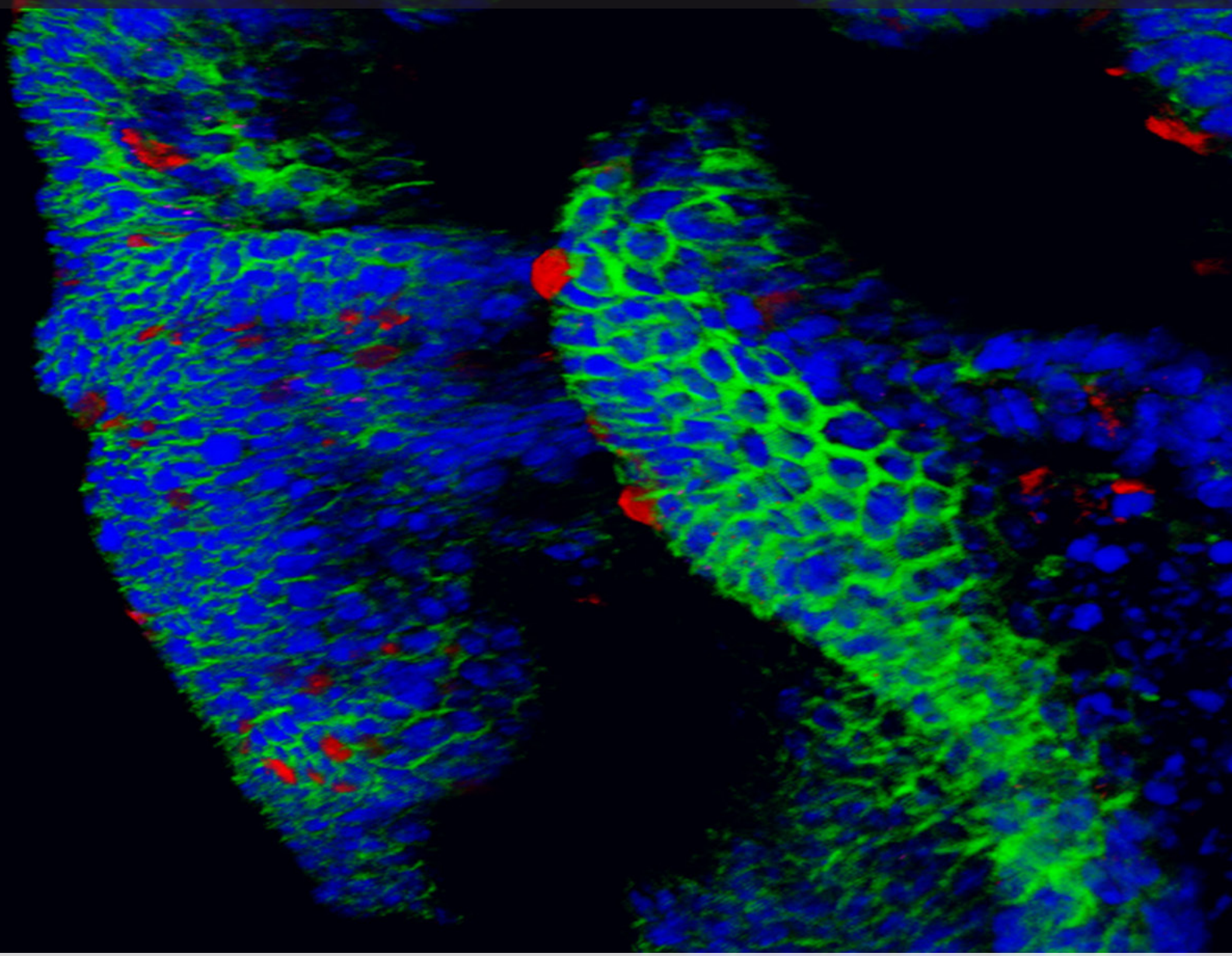
ORGANOGENESIS: FROM DEVELOPMENT TO DISEASE

EDITED BY: Sunder Sims-Lucas, Misty Good and Seppo Vainio
PUBLISHED IN: Frontiers in Cell and Developmental Biology



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ISSN 1664-8714
ISBN 978-2-88945-318-4
DOI 10.3389/978-2-88945-318-4

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ORGANOGENESIS: FROM DEVELOPMENT TO DISEASE

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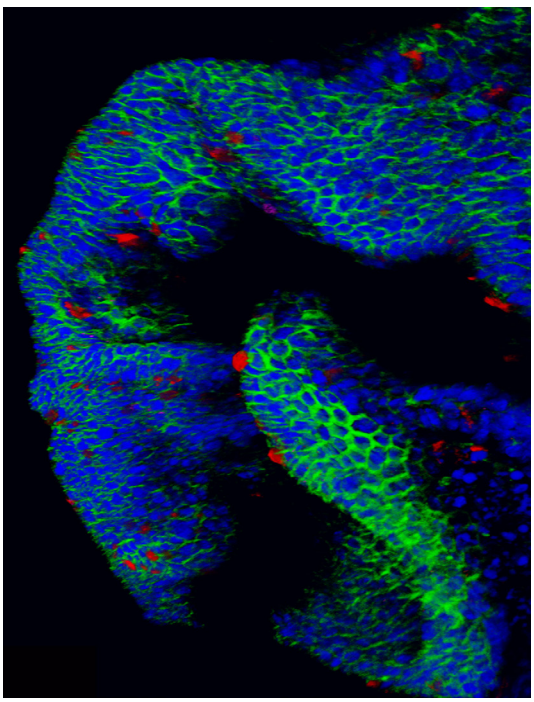

Confocal micrograph of mouse enteroid stained for the intestinal epithelial marker E-cadherin (Ecad-green), goblet cells (muc2-red), paneth cells (lysozyme-purple) or nuclear stain (DAPI-blue).

Image credit: Cliff Luke, PhD.

Citation: Sims-Lucas, S., Good, M., Vainio, S., eds. (2017). Organogenesis: From Development to Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-318-4

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frontiers in Cell and Developmental Biology

EDITORIAL
published: 20 September 2017
doi: 10.3389/fcell.2017.00085

Editorial: Organogenesis: From Development to Disease

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Keywords: animal, inflammation, kidney development, bowel disease, diabetes mellitus, type 1, bladder innervation, bone mineralization

Editorial on the Research Topic

Organogenesis: From Development to Disease

The formation of the developing fetus is under strict molecular and cellular control. These developmental processes have to coordinate seamlessly to produce a viable and healthy fetus. When these processes go astray this can lead to dramatic defects to the embryonic tissue and even to the death of the embryo. These miscues can be due to genetic or environmental influences. Even slight malformations of the organs can cause developmental reprogramming and increase the likelihood of adult onset diseases. The use of experimental animals with genetic mutations that mimic many human conditions has lead to significant scientific advancement of these complex and multifaceted diseases. Furthermore, the utilization of organ culture systems has made for profound insights into the formation of organs. Over recent years, the technology related to molecular profiling and imaging of developing organs has dramatically improved, leading to the identification of subtle genetic and phenotypic alterations. The major objectives of this topic was to explore the vast array of research that is focused on the formation of tissue and how this relates to the production of healthy tissue.

This editorial highlights the diverse nature of organ development and the exciting cross-disciplinary approaches that can be employed to answer questions related to the origin of developmental abnormalities, treatments, and post-natal susceptibility. This series of publications is made up of a combination of original science, reviews and mini-reviews.

The first original article describes the protective role of immunostimulated arginase expressing intestinal epithelial cells, which may be critical in the treatment of necrotiing enterocolitis (NEC). This is a highly important area of research as the incidence of NEC has not changed in the last several decades and the mortality rate can approach 50%. The treatment strategies to attenuate the exaggerated inflammation in this devastating disease are limited and the authors define an important mechanism that may be leveraged for therapeutic benefit (Talavera et al.).

The next original article focuses on establishing a mathematical model for bone mineralization (Komarova et al.). Mineralization is critical for the structural integrity of bone and without appropriate calcification the bones are fragile and prone to fractures and breaks. Up until now there has not been an effective characterization of bone mineralization. This manuscript has developed innovative algorithms to map the various stages of bone mineralization that may be applied to patients with genetic defects to aid in their personalized treatments.

There is a series of reviews on kidney formation including an article interrogating the multifaceted role of beta catenin in the kidney (Boivin et al.). Beta catenin signaling is one of the critical building blocks of kidney development and is an important signaling pathway involved in the pathogenesis of renal dysplasia. Together with the various Wnt ligands this signaling cascade is essential for almost every component of the developing kidney. The second kidney review focuses

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 August 2017*
Accepted: *06 September 2017*
Published: *20 September 2017*

Citation:
Sims-Lucas S, Good M and Vainio SJ (2017) Editorial: Organogenesis: From Development to Disease. Front. Cell Dev. Biol. 5:85. doi: 10.3389/fcell.2017.00085

on the current approaches to generate functional kidney tissue (Hariharan et al.). The field has been moving toward the generation of personalized therapies for each individual patient. This is largely driven by the generation of kidney cells and organoids from a patient's own cells. This review discusses the current state of stem cell reprogramming and differentiation toward a kidney fate. Finally, the last kidney review investigates the utilization of circulating exosomes as diagnostic markers and therapeutic agents (Krause et al.). Kidney disease leading to end stage kidney failure is one of the fastest growing disease processes and is further driven by the rising numbers of diabetic and obese patients. The therapies for kidney disease are limited and often the disease presents very late at an irreversible stage. This review details the exciting field of exosomes and describes their role in development and disease. Further, it discusses their utilization as a potential biomarker and vehicle for therapeutics.

Following is a review on the formation of the bladder, with a particular focus on the innervation of the bladder (Keast et al.). The primary function of the bladder is to store and excrete urine, which is controlled by the sympathetic and parasympathetic nervous systems. This is driven by a complex and detailed innervation pattern controlling the bladder muscle and the various genitourinary openings. This review details the innervation and localization of the neuronal bundles throughout development utilizing various rodent models.

There are two review articles that focus on the bowel the first looking at the role of the cytokine interleukin 22 (IL-22) (Parks et al.). Data is emerging regarding the precise role of IL-22 in the intestine. This review summarizes the current data on the role of IL-22 as an inhibitor of inflammation and also as a key player in the gut barrier defense against various pathogens. The second review focuses on the mitochondria in inflammatory bowel disease (Novak and Mollen). Inflammatory bowel disease is a complex and multifactorial disorder with unknown etiology. Typically, there is destruction of the epithelial layers of the gastrointestinal tract, increased permeability through these damaged layers and an influx of inflammatory cells. These epithelial cells are highly metabolically active and the primary engine of this energy source is the mitochondria. This review delves into the mitochondrial damage that is observed in inflammatory bowel disease and discusses whether this dysfunction is the cause or a readout of the damage in these patients.

The final review looks at the role of β cell endoplasmic reticulum (ER) stress in the context of Type 1 diabetes (Marre et al.). Type 1 diabetes is a chronic autoimmunity disorder as the β cells are damaged and unable to produce insulin, the glucose levels continue to climb leading to further damage. ER stress is a common attribute to the β cell as a part of its normal physiological role of insulin generation. This review highlights the specific role of ER stress in triggering the autoimmune response and suggests that this may be a sufficient mechanism in driving this disease progression.

This series of original articles and reviews has stimulated significant interest and impact in the field of organogenesis. It is exciting how each field draws parallels that can be applicable across the biomedical sciences and various disease processes. We would like to thank the authors for their valuable contributions and the thought provoking manuscripts that they have produced.

AUTHOR CONTRIBUTIONS

SS wrote the entire first draft of the manuscript and performed the editing before submission. MG and SV edited the first draft and provided intellectual input.

FUNDING

SS is supported by a K01 (DK096996) and R03 (DK110503). MG is supported by a K08 (DK101608) and an R03 (DK111473). SV is supported by the Center of Excellence Programme 2012–2017 from the Academy of Finland (grant numbers 251314, 284605), the European Community's Seventh Framework Programme (FP7/2007-2013; grant FP7-HEALTH-F5-2012-INNOVATION-1 EURenOmics 305608), and H2020 Marie Skłodowska-Curie Actions Innovative Training Network "RENALTRACT" Project ID 642937.

ACKNOWLEDGMENTS

We would like to thank the authors for their valuable contributions and the thought provoking manuscripts that they have produced.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 01 March 2017
doi: 10.3389/fcell.2017.00015

Immunostimulated Arginase II Expression in Intestinal Epithelial Cells Reduces Nitric Oxide Production and Apoptosis

Maria M. Talavera[1,2]*, *Sushma Nuthakki*[3], *Hongmei Cui*[1], *Yi Jin*[1], *Yusen Liu*[1,2] and *Leif D. Nelin*[1,2]

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Increased production of nitric oxide (NO) and subsequent local cytotoxicity to mucosal epithelial cells has been proposed as a putative mechanism involved in the development of necrotizing enterocolitis (NEC). Intestinal epithelial cells (IECs) metabolize L-arginine to either nitric oxide (NO) by NO synthase (NOS) or to L-ornithine and urea by arginase. L-ornithine is the first step in polyamine synthesis important for cell proliferation, while NO production can lead to apoptosis. We hypothesized that in IECs immunostimulation increases both NOS and arginase expression, and that arginase activity mitigates NO production and apoptosis. Rat intestinal epithelial cells (rIEC-6) were immunostimulated by either incubation with lipopolysaccharide (LPS) alone for 24 h or by incubation with conditioned media (CM) for 24 h. CM was obtained from RAW 264.7 cells (a macrophage cell line) treated with LPS (*E. coli* 0127:B8; 1 μg/ml) for 4 h. The rIEC-6 stimulated with LPS or with CM had significantly higher levels of inducible NOS (iNOS) protein, NO production, and arginase II protein than did the control cells. Direct LPS stimulation of rIEC-6 produced a less robust increase in iNOS expression and NO (represented as nitrite percent of control) than did CM stimulation. Inhibition of arginase using N^{ω} hydroxyl-L-arginine (NOHA) further increased stimulated NO production in rIEC-6. Viable cell numbers were significantly lower in CM stimulated cells after 24 h than in controls, and inhibition of arginase activity with NOHA resulted in a further significant decrease in viable cell numbers. We conclude that immunostimulated arginase expression of rIEC-6 cells tempers cytokine-induced iNOS-derived NO production and apoptosis.

Keywords: inducible nitric oxide synthase, arginase, inflammation, necrotizing enterocolitis

OPEN ACCESS

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 September 2016*
Accepted: *14 February 2017*
Published: *01 March 2017*

Citation:
Talavera MM, Nuthakki S, Cui H, Jin Y, Liu Y and Nelin LD (2017) Immunostimulated Arginase II Expression in Intestinal Epithelial Cells Reduces Nitric Oxide Production and Apoptosis. Front. Cell Dev. Biol. 5:15. doi: 10.3389/fcell.2017.00015

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants and a leading cause of neonatal morbidity and mortality (Lin and Stoll, 2006; Neu and Walker, 2011). The incidence among very low birth weight (VLBW) infants has remained constant at 5–7% for the past 30 years (Horbar et al., 2012). Mortality from NEC varies, depending on the amount of bowel involvement and the presence of comorbidities, with reports of up to 50% mortality

in patients requiring surgery for NEC (Lin and Stoll, 2006). The pathogenesis of NEC remains unclear; however, it is likely the result of a multifactorial process in a susceptible host. Of particular interest is the role of inflammation-induced vascular dysfunction and epithelial cytotoxicity in the development of NEC (MacKendrick et al., 1993; Ford et al., 1997; Nowicki et al., 2005).

Nitric oxide (NO) is the principal inhibitory neurotransmitter in the gut, endothelial-derived NO is involved in the local regulation of mucosal blood flow and inflammatory-derived NO is involved in the loss of mucosal integrity (Stark and Szurszewski, 1992; Alican and Kubes, 1996). Increased production of NO and subsequent local cytotoxicity to mucosal epithelial cells has been proposed as one of the putative mechanisms in NEC development (Ford et al., 1997; Nadler et al., 2000; Chokshi et al., 2008). NO is synthesized from L-arginine by NO synthase (NOS), of which there are three isoforms inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (Moncada, 1997). Of the three isoforms of NOS described, iNOS is not constitutively expressed, but induced at high levels during inflammation resulting in relatively high levels of NO production (Di Lorenzo et al., 1995; Chokshi et al., 2008). The role of iNOS-derived NO in the pathogenesis of NEC was first described by Ford and colleagues who demonstrated elevated iNOS expression in resected human NEC tissue compared to non-NEC control tissue (Ford et al., 1997). In experimental models of NEC, the inhibition of iNOS has been found to attenuate inflammatory intestinal injury (Ciftci et al., 2004; Giannone et al., 2006; Cintra et al., 2008).

L-arginine can be metabolized by arginase, of which there are two isoforms, arginase I, and arginase II. Arginase I has been described as the liver isoform, although it is found in many cell types throughout the body, while arginase II has been described as the inducible form of arginase and found in the kidney and small intestine (Badurdeen et al., 2015). Arginase converts L-arginine to L-ornithine, the first step in proline and polyamine synthesis, which are necessary for cell proliferation, collagen synthesis, and tissue regeneration. Alternatively, L-arginine metabolized by iNOS generates relatively high amounts of NO which can lead to cytotoxicity and apoptosis (Munder, 2009). We have previously found in endothelial cells and in macrophages that arginase and NOS compete for their common substrate, such that inhibiting arginase increased NO production (Chicoine et al., 2004; Jin et al., 2015). Therefore, we hypothesized that in intestinal epithelial cells inflammation would increase both NOS and arginase II protein expression, and that the inflammation-induced arginase activity acts to mitigate iNOS-derived NO production and thereby mitigates apoptosis. We used two different models of inflammation in these studies to induce iNOS and arginase. The first was direct treatment with lipopolysaccharide (LPS) to these cells as previously described (Talavera et al., 2015) and the second was to incubate the intestinal epithelial cells in conditioned media (CM) obtained from macrophages treated with LPS for 4 h. The LPS treatment gives insight into the direct effect of LPS on intestinal epithelial cells, while the CM, which would contain a mixture of cytokines and chemokines, provides insight into what may occur in the intestinal mileu during inflammation.

MATERIALS AND METHODS

Cell Culture

Rat intestinal epithelial cells, rIEC-6, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). rIEC-6 cells (studied in *passages* 15–20) are well-described, immortalized, immature, non-transformed rat small intestinal epithelial cells (Quaroni et al., 1979). Cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Salt Lake City, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 U/ml of recombinant human insulin. Cells were maintained in 100 mm tissue culture plates at 37°C in a humidified atmosphere with 5% CO_2 in ambient air (21% O_2). Cells were grown to a confluent monolayer prior to experimentation on 60 mm or 6-well plates (Thermo Fisher Scientific, Waltham, MA). Depending on the experiment, some wells were treated with LPS (*E. coli* 0127:B8; Sigma Chemicals) to a final media concentration of 100 μg/ml for 24 h. Other wells were treated with conditioned media (CM) for 24 h. The conditioned media was obtained by incubating a macrophage cell line (RAW 264.7 cells; American Type Culture Collection, Manassas, VA) with 1 μg/ml LPS (*E. coli* 0127:B8) for 4 h. The media was then harvested and placed on rIEC-6 cells for the indicated experiments as the immunostimulant.

Nitrite Assay

rIEC-6 cell media was collected in 1.5 ml tubes after treatment. The samples were assayed in duplicate for nitrite (NO_2^-) using a chemiluminescence nitric oxide analyzer (model 280i; Sievers Instruments, Boulder, CO) as previously described (Nelin et al., 2001; Chicoine et al., 2004). Briefly, 50 μl of sample were placed in a reaction chamber containing a mixture of sodium iodide (NaI) in glacial acetic acid to reduce NO_2^- to NO. The NO gas was carried into the NO analyzer by a constant flow of Helium gas. The analyzer was calibrated with a $NaNO_2$ standard curve.

Urea Assay

The samples of medium were assayed in triplicate for urea concentration colorimetrically as previously described (Chicoine et al., 2004). Briefly, 100 μl of sample was added to 1.5 ml of chromogenic reagent (5 mg thiosemicarbazide, 250 mg diacetyl monoxime, 37.5 mg $FeCl_3$ in 150 ml 25% (vol/vol) H_2SO_4, 20% (vol/vol) H_3PO_4). The mixtures were cooled to room temperatures and the absorbance (530 nm) was determined and compared with a urea standard curve.

RNA Isolation

RNA was isolated from rIEC-6 cells, as previously described (Talavera et al., 2015). Briefly, 0.7 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA) was added to cells and incubated for 5 min at room temperature. Cells were scraped and the mixture collected in 1.5 ml centrifuge tubes. Chloroform (0.1 ml) was added, the tubes shaken for 15 s and incubated at 30°C for 3 min.

The mixture was then centrifuged at 12,000 × g for 15 min at 4°C and the supernant collected. Isopropyl alcohol (0.25 ml) was added, the mixture was incubated at 30°C for 10 min and then centrifuged at 7,500 × g for 5 min at 4°C. The supernatant is then discarded, the pellet washed with 75% ethanol and centrifuged at 7,500 × g for 5 min at 4°C. The supernatant is again discarded, the pellet partially dried, dissolved in RNase-free water, and stored at −80°C.

Quantitative Real-time PCR

qPCR was performed as previously described (Talavera et al., 2015). Briefly, 4 μg of total RNA was pretreated with RQ1 RNase-free DNase (Promega) by incubating at 37°C for 30 min in a total volume of 10 μl. This reaction was terminated with the addition of RQ1 DNase stop solution. The reaction was then incubated at 65°C for 10 min to inactivate the DNase. The post-treated total RNA then underwent reverse transcription in a total volume of 40 μl containing 2.5 μM dT_{16} (Applied Biosystems, Foster City, CA), 20 units AMV-RT, 1 mM dNTP, 1x AMV RT buffer (Promega), and RNase-free water. The samples were incubated in a PCR-iCycler (Bio-Rad, Hercules, CA) at 42°C for 60 min, 95°C for 5 min, and stored at −20°C. Quantitative real-time PCR was performed with the Chromo 4 Real-time PCR Detection System (Bio-Rad), using qPCR SYBR Green Master-mix (Thermo Scientific). PCR reactions were performed for 40 cycles using the following parameters: 95°C for 15 s, 55°C for 30 s, 72°C for 30 s. The melting curves were manually verified for the presence of a single product. Arginase II was amplified using the forward primer (5′ AGAGAAGGCGGACACATTGCCTAT 3′) and the reverse primer (5′ TGTCGTGAAAGGTGCCAGAGT ACA 3′). iNOS was amplified using the forward primer (5′ TGTA GCCGCTGTGTGTCACAGAAGT 3′) and the reverse primer (5′ AGCAAAGGCACAGAACTGAGGGTA 3′).18S was amplified using the forward primer (5′ CCAGAGCGAAAGCATTTGCC A 3′) and the reverse primer (5′ TCGGCATCGTTTATGGTC GGAACT 3′). For each reaction, negative controls containing reaction mixture and primers without cDNA were performed to verify that primers and reaction mixtures were free of template contamination. Relative arginase II and iNOS transcripts were normalized to 18S expression using ΔΔ CT method (Livak and Schmittgen, 2001).

Protein Isolation

Protein was isolated from rIEC-6 cell lysate as previously described (Jin et al., 2010; Talavera et al., 2015). Briefly, rIEC-6 cells were washed with ice-cold phosphate-buffered saline (PBS), and 50–100 μl of lysis solution (0.2M NaOH, 0.2% SDS) was added to each plate or each well of a six-well plate. Thirty minutes before use the following protease inhibitors were added to each ml of lysis solution: 1 μl aprotinin [10 mg/ml in double distilled (dd) H_2O], l μl leupeptin (10 mg/ml in ddH_2O), and 1 μl of phenylmethylsulfonyl fluoride (34.8 mg/ml methanol). The rIEC-6 cells were scraped, collected into sterile centrifuge tubes, and placed on ice for 30 min. The cell lysates were centrifuged at 12,000 × g for 15 min. The supernatant was stored at −80°C. Total protein concentration was determined by the Bradford method (Bradford, 1976) using a commercially available assay kit (Bio-Rad, Hercules, CA).

Western Blot

Cell lysate was assayed for protein levels of 3-nitro-tyrosine (3-NT), proliferating nuclear antigen (PCNA), iNOS, arginase I, arginase II, and cleaved-caspase-3 using immunoblot analyses as previously described (Chicoine et al., 2004; Talavera et al., 2015). Briefly, aliquots of cell lysate were diluted 1:1 using SDS sample buffer, heated to 80°C for 15 min, and then separated using SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride membranes and blocked overnight in TBS with 0.1% Tween (TBS-T) containing 5% nonfat dried milk. The membranes were then incubated with primary antibody overnight; 3-nitrotyrosine (3-NT) (1:750; Sigma Aldrich, St. Louis, MO), PCNA (1:5000; Sigma Aldrich), arginase I (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), arginase II (1:500, Santa Cruz Biotechnology), cleaved caspase-3 (1:1,000, Cell Signaling Technology, Beverly, MA) or iNOS (1:1,000, BD BioSciences, San Jose, CA). The following day, membranes were washed three times with TBS-T, then incubated with horseradish peroxidase-conjugate goat anti-rabbit IgG (1:10,000, Bio-Rad) for 1 h, washed again three times with TBS-T. The protein bands were visualized using enhanced chemiluminescence (ECL plus reagent; Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using densitometry (Sigma Gel, Jandel Scientific, San Rafael, CA). To control for protein loading, the blots were stripped using a stripping buffer containing 62.5 mM Tris HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol, and the blots were reprobed for β-actin (1:10,000; Abcam, Cambridge, MA) as described above. Data are shown as the mean of the density of the protein of interest normalized to the density of β-actin.

Proliferation Assay

To determine cell proliferation, rIEC-6 cells were seeded in six-well plates at a density of 1×10^5 cells/well. The cells were incubated in regular DMEM or in CM for 24 h and viable cells counted using trypan blue exclusion. To determine the effects of arginase inhibition on rIEC-6 proliferation following immunostimulation, cells were incubated in CM with either vehicle or 100 μM N^{ω}-Hydroxy-nor-L-arginine (NOHA) added for 24 h and viable cells counted using trypan blue exclusion. In a separate set of proliferation experiments, using the same methodology as above, the effect of NOS inhibition on cell proliferation was determined by adding either vehicle or N^{ω}-nitro-L-arginine methyl ester (L-NAME) to CM, incubating the cells for 24 h, and then counting the viable cells using trypan blue exclusion.

Statistical Analysis

Values are expressed as means ± SEM. The groups were compared using a *t*-test when 2 groups were compared or a one-way analysis of variance (ANOVA) or two-way ANOVA when more than two groups were compared. For the ANOVA a Student-Newman-Keuls *post-hoc* test was used to identify significant differences. Differences were considered significant when $p < 0.05$ (Prism; GraphPad Software, San Diego, CA).

RESULTS

LPS-Induced Nitric Oxide Production in rIEC-6 Cells

To determine the effects of LPS on nitric oxide and urea production in enterocytes, cells were grown to ∼ 80% confluence, treated with LPS (*E. coli* 0127:B8) or left untreated (control) for 24 h. The cells were harvested for RNA and analyzed by qRT-PCR. There was increased iNOS mRNA levels ($p < 0.05$) from LPS stimulated cells as compared with unstimulated IECs (**Figure 1A**). Cell lysates were collected and evaluated by Western blot for iNOS protein levels. The LPS-treated cells showed ∼7-fold greater iNOS protein expression than control ($p < 0.001$) (**Figure 1B**). In a similar set of experiments, the cell media was examined for production of nitrites as a marker of NO production. LPS-treated cells had substantially greater ($p < 0.001$) NO concentration (represented as percent control) than did control cells (**Figure 1C**).

To examine LPS effects on arginase, cell lysates were assayed for arginase I and arginase II mRNA and protein levels. There was no significant effect of LPS on arginase I mRNA or protein levels in these rIEC-6 cells (data not shown). However, LPS-treated cells had substantially greater levels of arginase II mRNA (**Figure 1D**) and arginase II protein (**Figure 1E**) than did control cells. The cell media was also assayed for urea production. The LPS-treated rIEC-6 cells significantly greater ($p < 0.001$) urea concentration (represented at percent of control) that did vehicle-treated cells (**Figure 1F**).

LPS-induced Nitrite Production Is Enhanced by Arginase Inhibition

To determine the role of arginase activity in LPS-induced NO production in rIEC-6 cells we utilized the putative arginase inhibitor NOHA. Cells were either not treated or treated with LPS and the LPS-treated cells were also treated with vehicle or NOHA (100 μM). The media was collected 24 h later and assayed for nitrites and urea. LPS-treated cells had significantly greater urea production than control cells and NOHA treatment attenuated the LPS-induced increase in urea production (**Figure 2**). LPS-treated cells had substantially greater NO production than did control cells and treatment with NOHA augmented LPS-induced NO production (**Figure 2**). These findings are consistent with the concept that arginase acts to limit iNOS-derived NO production in LPS stimulated rIEC-6 cells.

Arginase Inhibition Results in Greater Cytotoxicity and Decreased Proliferation in LPS-treated rIEC-6 Cells

To examine the effects of arginase inhibition on NO-mediated cytotoxicity in LPS-treated rIEC-6 cells, we performed Western blot analyses for 3-nitrotyrosine—(3-NT) protein expression. 3-NT is a marker for peroxynitrite formation, which is formed in the presence of NO and superoxide during conditions of oxidative stress. LPS-treatment resulted in a significant increase in 3-NT level and treatment with LPS + NOHA resulted in greater 3-NT levels than in LPS-treated cells (**Figure 3A**). Inhibtion of arginase in control cells also led to a significant production of 3-NT but not significantly more than arginase inhibition following LPS stimulation ($p < 0.001$) (**Figure 3A**).

We also examined protein levels of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation in each experimental group, hypothesizing that if NO production were greater in the LPS treated cells that PCNA levels would be lower and that arginase inhibition would further lower PCNA protein levels. We found a significantly lower level of PCNA protein in LPS-treated cells than in control cells (**Figure 3B**). Furthermore, when arginase was inhibited in the LPS-treated cells the levels of PCNA were lowest (**Figure 3B**). These results indicate that arginase inhibition in LPS-treated enterocytes lead greater nitrosative damage and decreased cell proliferation.

CM Increases iNOS and Arginase II Protein Levels

To examine the effect of another form of immunostimulation on iNOS and arginase II protein levels rIEC-6 cells were treated with either CM or regular media (control) for 24 h. Cell lysates were examined for iNOS and arginase II protein levels by Western blot analysis. Following CM stimulation, iNOS protein levels were substantially higher than in control cells (**Figure 4A**). The fold increase in iNOS protein levels with CM relative to control were substantially greater than the fold increase in iNOS protein levels with LPS stimulation alone. Similarly, in cells incubated in CM the arginase II protein levels were significantly higher than in the control cells (**Figure 4B**). Interestingly, the fold change in arginase II levels relative to control caused by CM was similar to that seen with LPS.

Treatment with CM Increased Apoptosis in rIEC-6 Cells

To begin to examine the effects of iNOS induction on cell viability, the protein levels of cleaved caspase-3 were determined in rIEC-6 cells following incubation in CM. The cleaved caspase-3 levels were nearly 10-fold greater ($p < 0.0001$) in rIEC-6 cells incubated in CM compared to control cells (**Figure 5**). This data suggest that the CM-induced increase in iNOS protein levels in rIEC-6 cells was associated with the greater apoptosis.

Inhibition of Arginase Results in Greater NO Production and Fewer Viable Cells

To determine the effects of CM and arginase inhibition on rIEC-6 NO production, rIEC-6 cells were grown to 80% confluence and then incubated in CM or regular media with either vehicle or 100 μM NOHA added for 24 h. Nitrite levels were measured in the media. Similar to our findings with LPS alone, treatment with CM resulted in a substantially greater nitrite levels than in control cells (**Figure 6A**). Inhibiting arginase with NOHA in the CM-treated cells resulted in substantially greater nitrite levels than in the CM and vehicle treated cells (**Figure 6A**).

To determine the effect of CM on viable cell number, rIEC-6 cells were seeded at a density of 1×10^5 cells/well in a six-well plate. These cells were then treated with regular media (control), CM or CM with 100 μM NOHA for 24 h and viable cell numbers

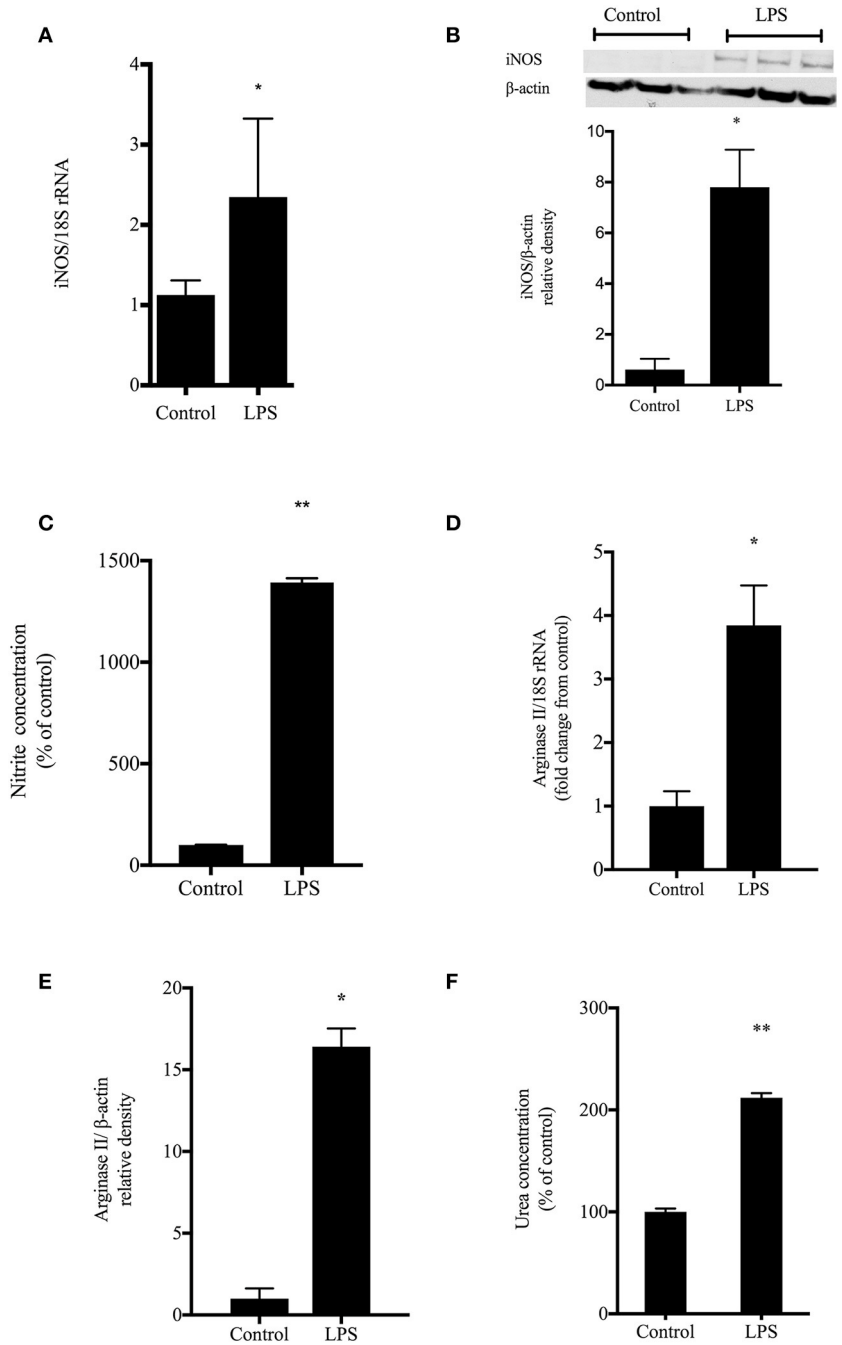

FIGURE 1 | LPS induced NO and urea production. rIEC-6 cells were treated with LPS or vehicle (control) for 24 h. **(A)** iNOS mRNA levels detected by qRT-PCR normalized to 18srRNA levels ($n = 6$ for each group). **(B)** Cell lysates were harvested and iNOS protein levels determined by Western blot. The densitometry data ($n = 3$ for each group) normalized to β-actin are shown relative to control. **(C)** Nitrites were analyzed by chemiluminescence for media harvested after a 24 h incubation with vehicle (control) or LPS ($n = 9$ for each group). Nitrite levels were measured in media and represented as a percent of control. **(D)** Cell lysates were examined for arginase II mRNA levels using qPCR normalized to 18s rRNA ($n = 6$ for each group). Densitometry results are shown as fold change from control (where control levels = 1). **(E)**. Cell lysates were examined for arginase II protein levels by Western blot ($n = 4$ for each group). The densitometry data normalized to β-actin are shown relative to control. **(F)**. Urea production was determined by colorimetric assay after harvesting medium from cells treated with vehicle (control) or LPS ($n = 6$ for each group). Urea levels were measured in media and are represented as a percent of control urea values. *LPS different from control, $p < 0.05$, **LPS different from control, $p < 0.001$.

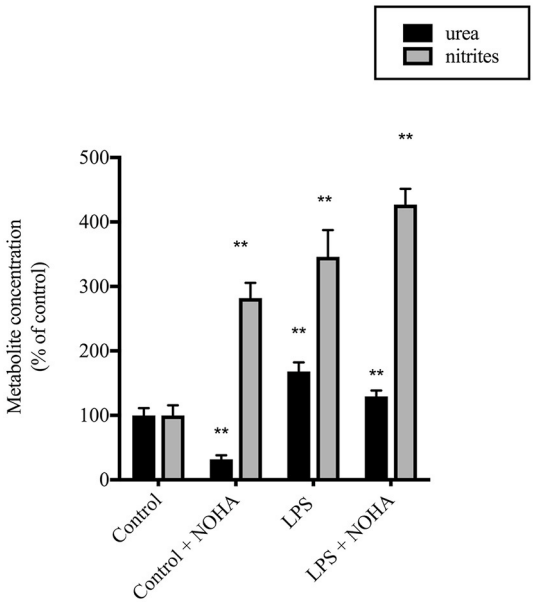

FIGURE 2 | LPS-induced NO production was augmented when urea production was inhibited. rIEC-6 cells were incubated for 24 h in media containing LPS and either vehicle or 100 μM N$^{\omega}$-Hydroxy-nor-L-arginine acetate (NOHA). Urea (black bars) was measured using a colorimetric assay and the results are presented as percent of control (i.e., no LPS) urea values. Nitrites (gray bars) were measured in the media and are presented as a percent of control nitrite values. **LPS different from control; **LPS+NOHA different from control + NOHA, $p < 0.001$; multiple *t*-test and 2-way ANOVA.

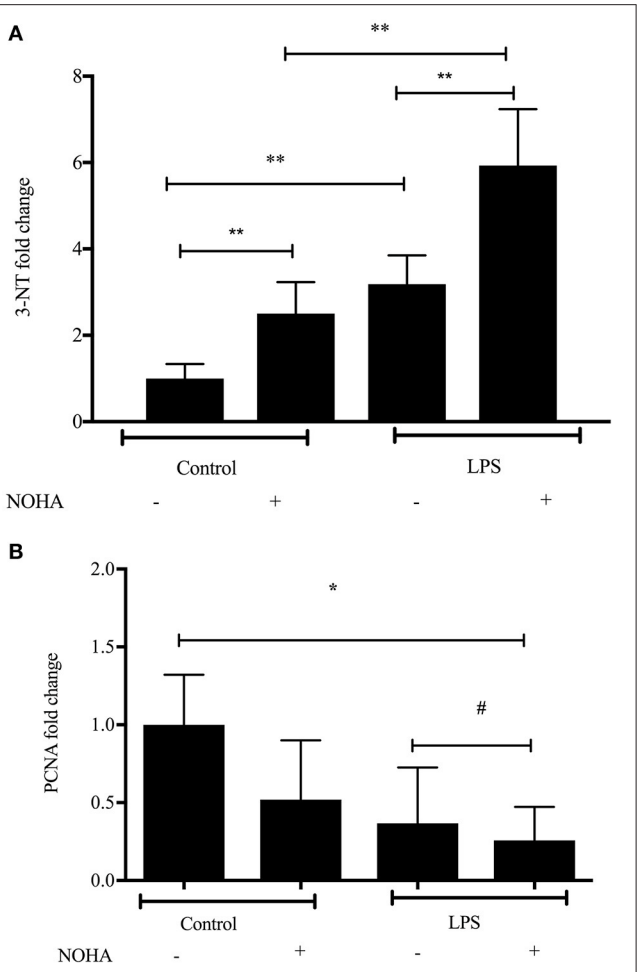

FIGURE 3 | Arginase inhibition resulted in increased 3-nitrotyrosine and decreased proliferation in LPS-treated rIEC-6 cells. rIEC-6 cells were treated with LPS or untreated (control), and either with the arginase inhibitor (NOHA) or its vehicle for 24 h. Cell lysate was examined for 3-NT, PCNA or β-actin by Western blot. **(A)**. Densitometry data for 3-NT normalized to β-actin and presented as fold change from control ($n = 6$ for each group); **LPS+ NOHA-treated different from control + NOHA; LPS + NOHA different from LPS alone; LPS different from control; Control + NOHA different from control, $p < 0.001$. **(B)**. Densitometry data for PCNA normalized to β-actin and presented as fold change from control ($n = 9$ for each group); #LPS different from LPS+NOHA control, $p < 0.05$. *LPS+NOHA different from control, $p < 0.001$.

determined using trypan blue exclusion. Treatment of rIEC-6 cells with CM resulted in significantly fewer viable cells than in control rIEC-6 cells (**Figure 6B**). Addition of NOHA to the CM resulted in significantly fewer viable cells than in the cells incubated in CM and vehicle (**Figure 6B**). These findings support a role for arginase in attenuating iNOS generated NO production to preserve rIEC-6 cell viability following inflammatory stimuli.

Inhibiting Arginase Augments Apoptosis, While Inhibiting NOS Attenuates Apoptosis

To determine the role of arginase inhibition on enterocyte apoptosis, rIEC-6 cells were grown to ∼80% confluence then stimulated with either CM or CM + NOHA (100 μM) for 24 h. Cell lysate was collected and protein harvested. Cleaved caspase-3 protein levels were determined by Western blot analyses. We found significantly greater cleaved caspase- 3 protein levels in the rIEC-6 cells treated with CM + NOHA than in cell treated with CM alone (**Figure 7A**). To determine the role of immunostimulated NO production on enterocyte apoptosis, rIEC-6 were incubated in either CM or CM + L-NAME (100 μM) for 24 h. Cell lysates was collected and protein harvested. Cleaved-caspase-3 protein levels were determined by Western blot analyses. The CM + L-NAME treated cells had significantly lower ($p < 0.001$) cleaved caspase-3 protein levels than did cells incubated in CM alone (**Figure 7B**). These results suggest that during intestinal inflammatory conditions arginase expression in enterocytes may temper iNOS-mediated apoptosis.

DISCUSSION

The major findings of this study in rIEC-6 were that: (1) immunostimulation increased iNOS and arginase II protein levels, as well as NO and urea production; (2) inhibiting arginase resulted in greater immunostimulated NO production; (3) immunostimulation increased apoptosis; (4) inhibition of arginase resulted in further decreased viable cell number and increased apoptosis following immunostimulation; and (5) inhibition of NOS production attenuated apoptosis following immunostimulation. These findings support our hypothesis that immunostimulation of intestinal epithelial cells increased both iNOS and

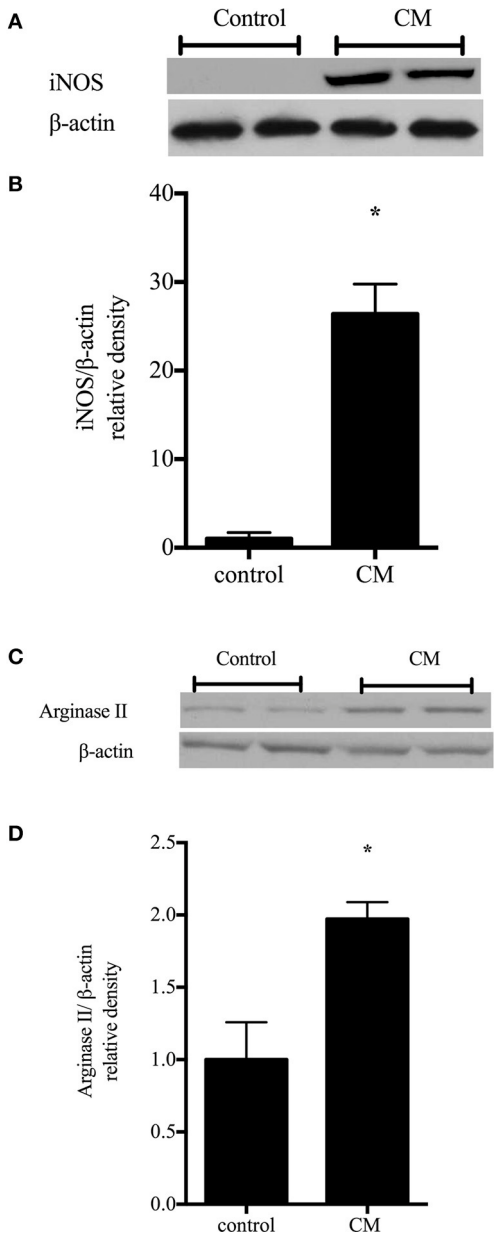

FIGURE 4 | Conditioned media up-regulates iNOS and arginase II protein levels. rIEC-6 cells were exposed to conditioned media (CM) for 24 h. CM media (DMEM with FBS) was collected from RAW 264.7 cells stimulated with 1 μg/ml LPS for 4 h. **(A)** Cell lysates ($n = 6$ for each group) were examined for protein levels of iNOS by western blotting. **(B)** The densitometry data normalized to β-actin are shown relative to control. **(C)** Cell lysates ($n = 6$ for each group) were examined for protein levels of arginase II by western blotting. **(D)** The densitometry data normalized to β-actin are shown relative to control. *CM different from control, $p < 0.005$.

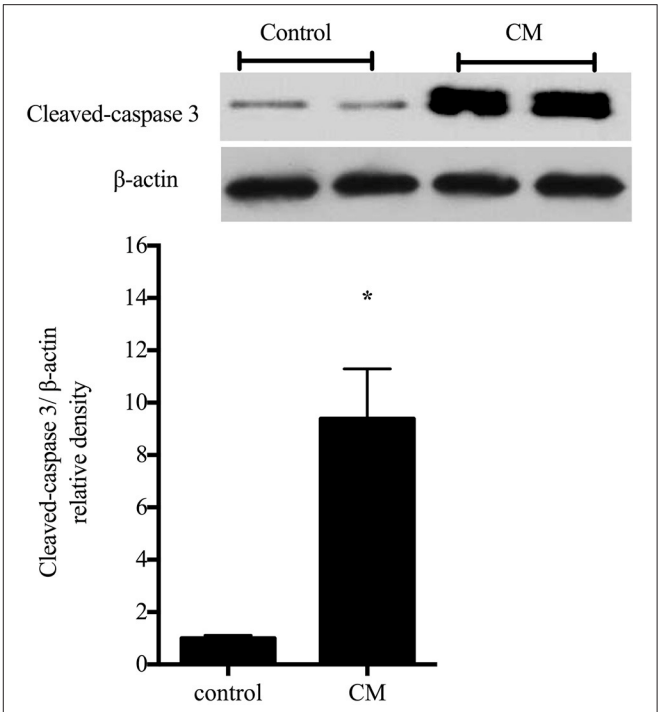

FIGURE 5 | Condtioned media increased apoptosis in rIEC-6 cells. Cells were exposed to conditioned media (CM) for 24 h. CM media (DMEM and FBS) was collected from RAW 264.7 cells stimulated with 1 μg/ml LPS for 4 h. Cell lysates were examined for levels of cleaved caspase-3 by western blotting. Typical western blots for cleaved caspase-3 and β-actin. The bar graph shows the densitometry data for cleaved caspase 3 normalized to β-actin ($n = 6$ for each group) are shown relative to control (i.e., control = 1). *CM different from control, $P < 0.005$.

arginase II protein expression and that arginase activity mitigated iNOS-derived NO production and subsequent apoptosis.

In this study we used two methods of experimental immunostimulation of rIEC-6 cells: direct LPS stimulation and incubation with conditioned media from LPS-stimulated macrophage cell line. The conditioned media would be expected to contain biologically relevant levels of a wide-variety of chemokines and cytokines. Intestinal epithelial cells (IECs) are in nearly constant contact with bacteria and bacterial products (LPS) and during inflammatory states there can be significant influx of inflammatory cells including macrophages (De Plaen, 2013). IEC's express cytokine receptors and secrete cytokines in response to inflammatory stimuli (Jung et al., 1995; Reinecker and Podolsky, 1995). We have previously shown that rIEC-6 cells express both TNF-α and COX-2 following direct LPS stimulation (Talavera et al., 2015). In this study, we found that both direct LPS and cytokine-induced (CM) immunostimulation resulted in greater expression of both arginase II and iNOS in rIEC-6 cells. Our data supports the notion that enterocyte-derived iNOS is up-regulated during inflammatory states and is responsible for high levels of NO production (Grishin et al., 2016).

These high levels of NO production can exert detrimental effects on the gut barrier leading to increased permeability, increased bacterial translocation (Hackam, 2011), impaired mitochrondrial function (Erusalimsky and Moncada, 2007), and impaired epithelial restitution following injury (Cetin et al., 2007). We found that enterocyte-derived iNOS expression in response to both LPS and conditioned media conditions

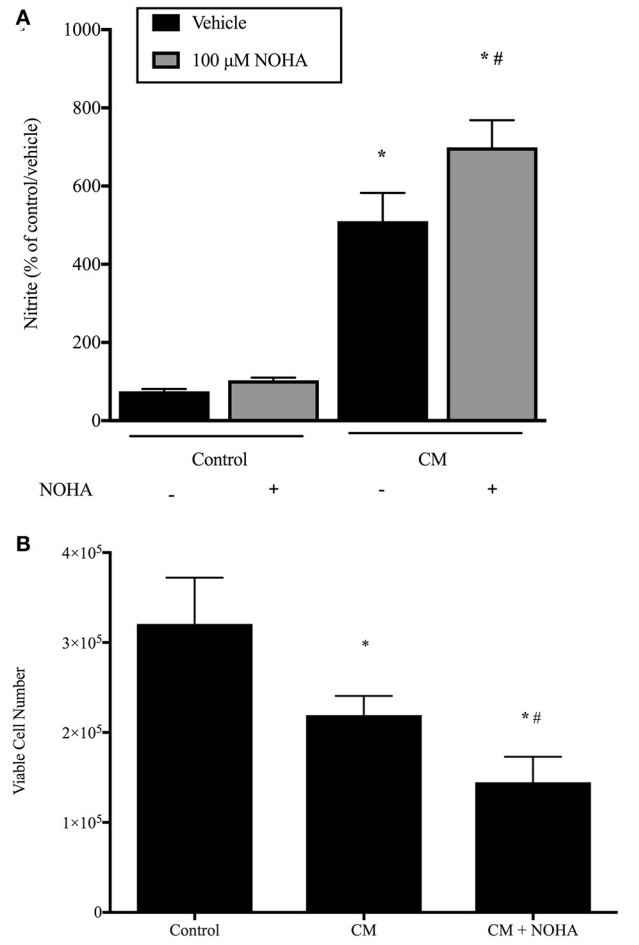

FIGURE 6 | Conditioned media results in more NO production and fewer viable cells and inhibition of arginase augments this response. rIEC-6 cells were incubated in either regular media (control) or conditioned media (CM), and with either vehicle or 100 μM NOHA added for 24 h. **(A)**. Media was harvested for determination of NO production by assaying nitrites using chemiluminescence. Conditioned media resulted in a substantially greater NO production in vehicle treated cells (black bars) than did regular media (control). Inhibiting arginase with NOHA (gray bars) augmented the CM-induced production of NO. *CM different from control, $p < 0.001$. # NOHA different from vehicle, $p < 0.005$. **(B)**. Equal numbers of rIEC-6 cells were seeded in each well of 6 well plates and treated as above. After 24 h viable cell numbers were determined using trypan blue exclusion. *CM different from control, $p < 0.05$. #CM + NOHA different from CM, $p < 0.05$.

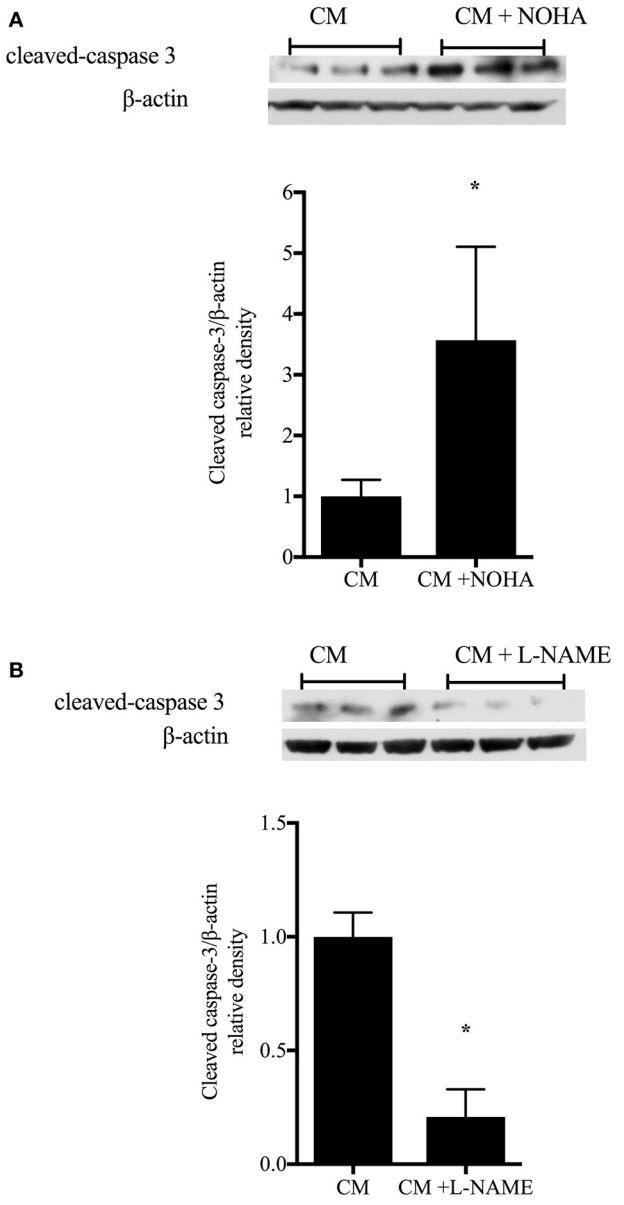

FIGURE 7 | Inhibition of arginase augments, while inhibition of iNOS attenuates, enterocyte apoptosis. rIEC-6 cells were grown ~80% confluence then incubated in either conditioned media (CM) alone or CM and the arginase inhibitor, NOHA **(A)**, or CM alone or CM + the iNOS inhibitor, L-NAME **(B)**, for 24 h. Cell lysates were collected and protein harvested to determine cleaved caspase-3 and β-actin protein levels. **(A)**. Representative Western blots for cleaved caspase-3 and β-actin in CM and CM + NOHA-treated cells ($n = 9$ each group). The bar graph is the densitometry data for cleaved caspase-3 normalized to β-actin and shown relative to CM alone. *$p < 0.05$ CM + NOHA different than CM alone. **(B)**. Representative Western blots for cleaved caspase-3 and β-actin in CM and CM + L-NAME-treated cells ($n = 9$ for each group). The bar graph is the densitometry data of cleaved caspase-3 normalized to β-actin as shown relative to CM alone. *$p < 0.001$ CM + L-NAME different than CM alone.

parallel elevated NO levels (**Figures 1**, **3**) and cleaved caspase-3 (**Figure 4**) expression. Cytoplasmic NO readily and very rapidly reacts with superoxide to form peroxynitrite, which is cytotoxic to enterocytes by the formation of nitrotyrosine residues alterating protein enzymatic activity (Schopfer et al., 2003; Grishin et al., 2016). We have shown that direct LPS stimulation following arginase inhibition significantly increased 3-nitrotyrosine levels and decreased PCNA levels in rIEC-6 cells. We also demonstrated that in the presence of a small molecule arginase inhibitor, NOHA, iNOS-dependent NO production was increased in rIEC-6 cells, and that this increase in NO production resulted in fewer viable cells. Thus, our data supports a central role of iNOS in enterocyte apoptosis, which would be expected to lead to decreased barrier function *in vivo*.

The opposing biological effects of iNOS and arginase in response to inflammation are mainly due to the cytopathic effects of NO compared to the regenerative, proliferative effects of arginase on the cellular environment (Hibbs, 1991; Chokshi et al., 2008). Others have shown how specific inflammatory ligands will determine a predominant iNOS or arginase expression. For example, in murine macrophages Th2 cytokines (IL-4 and IL-10) are potent inducers of arginase, whereas Th1 cytokine -INF-γ is a potent inducer of iNOS (Modolell et al., 1995). Similarly, in mouse peritoneal exudate cells, the growth factor, TGF-β, attenuated INF-γ-induced increased iNOS activity resulting in increased arginase activity (Shearer et al., 1997). In this study, we demonstrated that direct LPS stimulation of rIEC-6 cells resulted in arginase II induction and increased urea production. Concurrently, direct LPS stimulation also induced iNOS activity and increased NO production. It is unclear if iNOS and arginase II induction following either LPS or CM stimulation results from similar signaling cascades or if different signaling cascades are involved. In RAW cells, we recently reported that extracellular-signal regulated kinase (ERK) was involved in arginase II induction but that p38 was necessary for iNOS induction following LPS stimulation (Jin et al., 2015). Further studies will be needed in enterocytes to determine if similar cellular mechanisms are involved in immune-mediated iNOS and arginase II induction.

The dynamic interplay between iNOS and arginase activities has been reported in other cell types including vascular endothelium, macrophages and gastrointestinal epithelium (Eckmann et al., 2000; Berkowitz et al., 2003; Chicoine et al., 2004; Miki et al., 2009; Jin et al., 2015). In this report we studied the relative roles of immunostimulated iNOS and arginase II in intestinal epithelial cell NO production. We demonstrated that treatment with conditioned media (CM) induced a significantly greater iNOS expression compared to arginase II in rIEC-6 cells. We also demonstrated that cytokine-induced nitrite production was increased by arginase inhibition in rIEC-6 cells. These findings are consistent with a previous study in LPS/TNF-α-stimulated endothelial cells, wherein inhibition of arginase resulted in an increase in NO production (Chicoine et al., 2004). Our lab has also demonstrated that when LPS-induced arginase II expression in RAW 264.7 cells was prevented using a siRNA against arginase II, iNOS-derived NO production was substantial enhanced (Jin et al., 2015). These findings suggest that following immunostimulation in various cell types including enterocytes, arginase and iNOS compete for their common substrate, L-arginine, such that decreasing arginase activity leads to an increase in NO production. Thus, arginase activity is involved in cellular regulation of inflammatory NO production in intestinal epithelial cells.

Enhanced apoptosis in intestinal epithelial cells is a hallmark of intestinal barrier dysfunction, which is thought to be an important pathogenic feature for NEC in preterm infants (De Plaen, 2013). The increased enterocyte apoptosis observed in NEC has been shown to correlate with an increase in iNOS activity and 3-NT staining (Nadler et al., 2000). In this study, we demonstrated that inhibition of iNOS-induced NO production attenuated apoptosis in rIEC-6 cells, as evidenced by substantially lower levels of cleaved caspase-3 in cells treated with a NOS inhibitor, L-NAME. While augmenting iNOS-derived NO production by arginase inhibition resulted in significantly greater levels of cleaved caspase-3. These findings highlight the cytotoxic effects of iNOS-derived NO in rIEC cells and the role of arginase activation in attenuating inflammation-induced NO-mediated apoptosis.

In conclusion, we demonstrated that inflammation-induced NO production in rIEC-6 is iNOS mediated and leads to cytotoxicity and decreased viable cell numbers. We found that inflammation-induced arginase activity in enterocytes mitigates the effects of iNOS-derived NO production by attenuating NO production and the resultant apoptosis. These findings suggest that inhibition of iNOS and/or augmentation arginase II activities in enterocytes may be viable therapeutic strategies to prevent epithelial cell apoptosis and loss of barrier function. It is important to remember though that some level of NO is needed to maintain mucosal homeostasis, vascular tone, and oxidative stress, while relatively high levels of NO, as seen with iNOS induction, lead to apoptosis. Our findings suggest a potential therapeutic target would be augmented arginase activity in inflamed intestinal epithelial cells as a means to prevent disease progression and facilitate early proliferation and repair. We further speculate that neonates with lower degrees of arginase induction, perhaps due to genetic mutations in their arginase II genes, may be more prone to develop NEC.

AUTHOR CONTRIBUTIONS

MT, SN, YL, and LN contributed to the conceptualization, data analysis and manuscript preparation. SN, HC, and YJ contributed to conducting studies and data collection. All authors contributed to the conceptualization and organization of the manuscript as well as final manuscript approval.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 21 August 2015
doi: 10.3389/fcell.2015.00051

Mathematical model for bone mineralization

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Defective bone mineralization has serious clinical manifestations, including deformities and fractures, but the regulation of this extracellular process is not fully understood. We have developed a mathematical model consisting of ordinary differential equations that describe collagen maturation, production and degradation of inhibitors, and mineral nucleation and growth. We examined the roles of individual processes in generating normal and abnormal mineralization patterns characterized using two outcome measures: mineralization lag time and degree of mineralization. Model parameters describing the formation of hydroxyapatite mineral on the nucleating centers most potently affected the degree of mineralization, while the parameters describing inhibitor homeostasis most effectively changed the mineralization lag time. Of interest, a parameter describing the rate of matrix maturation emerged as being capable of counter-intuitively increasing both the mineralization lag time and the degree of mineralization. We validated the accuracy of model predictions using known diseases of bone mineralization such as osteogenesis imperfecta and X-linked hypophosphatemia. The model successfully describes the highly nonlinear mineralization dynamics, which includes an initial lag phase when osteoid is present but no mineralization is evident, then fast primary mineralization, followed by secondary mineralization characterized by a continuous slow increase in bone mineral content. The developed model can potentially predict the function for a mutated protein based on the histology of pathologic bone samples from mineralization disorders of unknown etiology.

Keywords: bone histomorphometry, matrix mineralization, mineralization inhibitors, nucleating centers, osteogenesis imperfecta, osteomalacia, X-linked hypophosphatemia, rickets

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *18 June 2015*
Accepted: *04 August 2015*
Published: *21 August 2015*

Citation:
Komarova SV, Safranek L, Gopalakrishnan J, Ou MY, McKee MD, Murshed M, Rauch F and Zuhr E (2015) Mathematical model for bone mineralization. Front. Cell Dev. Biol. 3:51. doi: 10.3389/fcell.2015.00051

Background

Defects in bone mineralization can result in reduced or excessive bone mineralization, which can lead to serious clinical manifestations, including bone deformities and fractures. Plasma levels of calcium and phosphate—ionic mineral constituents of bone hydroxyapatite mineral—as well as their key regulators parathyroid hormone, vitamin D and FGF23, are critically important for successful mineralization (Shimada et al., 2004; Morris et al., 2012). Numerous conditions which are not associated with abnormal levels of circulating calcium and phosphate are also known to result in hypo- or hypermineralization of bone matrix (Roughley et al., 2003).

One example is osteogenesis imperfecta, a disease usually caused by mutations in collagen type I-encoding genes and characterized by increased bone mineralization (Roschger et al., 2008a; Forlino et al., 2011). The clinical phenotype of osteogenesis imperfecta can also be caused by mutations in genes encoding the proteins that are involved in collagen post-translational modifications such as bone morphogenetic protein 1 (BMP1), or the proteins that regulate bone mineralization by an as yet unknown mechanism (Marini et al., 2014). The mechanisms underlying the development of hypo- and hypermineralization of extracellular bone matrix when the plasma levels of calcium and phosphate are within the normal range are complex and not well understood.

Clinically, the mineralization process can be examined in bone biopsy samples, which are typically obtained from the iliac bone. When tetracycline labeling is performed prior to biopsy, it is possible to assess the mineralization process quantitatively using bone histomorphometry (Rauch, 2006). Key, well-accepted histomorphometric descriptors of the mineralization process include the average thickness of the layer of unmineralized organic bone matrix (osteoid thickness) and the duration of the lag time between the deposition of organic matrix and the start of mineralization (mineralization lag time). Using quantitative backscattered-electron imaging, it is also possible to determine the average density of mineralized bone (Roschger et al., 2008b), which among other measures indicates the proportion of the mineralized tissue mass contributed to by the mineral ions.

The molecular origin and mechanistic basis of bone hypo- and hypermineralization are incompletely understood; however, it is clear that the process of mineralization is tightly regulated and is highly nonlinear. The goal of this study was to develop a simplified mathematical model of a complex process that provides a description of basic steps in the mineralization process, including collagen production and maturation, delivery and degradation of inhibitors, as well as mineral nucleation and growth. To our knowledge, no mathematical model focused on bone mineralization process exists to date, however important work has been performed in modeling the role of mineralization process in determining the bone mineral distribution (Ruffoni et al., 2007); the process of dentinogenesis including dentin phosphoprotein-regulated mineralization (Niño-Barrera et al., 2013) as well as describing the mineralization-induced changes in mechanical properties of collagen (Crolet et al., 2005; Nikolov and Raabe, 2008; Barkaoui and Hambli, 2014). Using the intentionally simplified model which includes multiple complexities in a limited number of variables, we were able to capture the significant nonlinearity of the mineralization process. Modeling predictions regarding the roles of individual processes in generating abnormal mineralization patterns were compared to the phenotype of diseases having in major bone mineralization defects—namely osteomalacia diseases and osteogenesis imperfecta.

Model Development

We modeled the changes over time in the concentration of five key players in the mineralization process—the collagen matrix subdivided into naïve and mature matrix, the inhibitors of mineralization, the nucleation centers (nucleators), and the hydroxyapatite mineral—within a homogeneous unit volume of osteoid of $\sim 1\,\mu m^3$ in dimensions using a system of ordinary differential equations. The following considerations were used to identify the main model assumptions.

1. Physiologically, the formation of bone tissue begins with the secretion of an organic bone matrix by osteoblasts, which to a large extent (by weight and volume) consists of collagen type I (Christiansen et al., 2000). Once this naïve organic matrix is deposited into the extracellular compartment, it needs to be processed in order to accommodate mineralization—a process termed matrix maturation. This maturation phase includes cleavage of C- and N-terminal propeptides from the collagen molecule and collagen crosslinking and packaging (Knott and Bailey, 1998; Christiansen et al., 2000), as well as similar processing of noncollagenous proteins (Kaartinen et al., 2002). The process of extracellular matrix maturation lasts 10–14 days. For this model, we considered different steps of matrix maturation such as post-translational modification of collagen and noncollagenous matrix proteins, and collagen cross-linking as a maturation process, which normally occurs with a characteristic rate constant of k_1. In the model, a change in any step of post-translational modification or crosslinking is assumed to have an overall effect on matrix maturation by either facilitating or interfering with the normal process. We assumed that collagen matrix is produced by osteoblasts in a naïve form (x_1) that matures into a fully assembled mature collagen matrix (x_2). These relationships are described by Equations (1a) and (1b).
2. The mineralization of naïve matrix is prevented by the action of numerous inhibitors, which reside in the local bone extracellular microenvironment, or arrive from the circulation (Murshed and McKee, 2010). During matrix maturation, the inhibitors are degraded or inactivated to enable mineralization. For example, a potent small-molecule inhibitor of mineralization—inorganic pyrophosphate—is cleaved by alkaline phosphatase present on the osteoblast cell membrane (Murshed and McKee, 2010). The proteins of the SIBLING family (small integrin-binding ligand, N-linked glycoproteins) are also known for their role in the regulation of mineralization; for example, the SIBLING proteins osteopontin (OPN) and matrix extracellular phosphoglycoprotein (MEPE) are potent inhibitors of mineralization (Rowe et al., 2004; Jahnen-Dechent et al., 2008). The action of another SIBLING protein dentin matrix protein 1 (DMP1) is regulated by its state during matrix maturation. DMP1 acts an inhibitor of mineralization when it is in solution (He et al., 2005), but becomes a promoter of mineralization when absorbed onto collagen surfaces (Hunter and Goldberg, 1993; Hunter et al., 1996; He et al., 2003). We modeled the combined action of different inhibitors of mineralization (I), which were assumed to be released into the extracellular compartment near the cells (Murshed and McKee, 2010) and diffuse through immature collagen (Weinstock and Leblond, 1973) with the characteristic rate

constant of v_1. Thus, inhibitor availability was modeled to be proportional to the amount of naïve collagen as described by the term $v_1 x_1$ in the Equation (1c). The concentration of active inhibitors in mineralizing matrix gradually decreases both because of their degradation through enzymatic cleavage (Addison et al., 2008, 2010; Murshed and McKee, 2010; Barros et al., 2013) as well as removal by other processes that interfere with inhibitor function, such as binding, masking or trapping (He et al., 2005; David et al., 2011). In the model, inhibitor removal/reduction occurs with the rate constant of r_1 and is stimulated by the presence of mature collagen matrix and as described by the term $r_1 x_2 I$ in the Equation (1c).

3. During the mineralization process calcium and phosphate precipitate to form hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ crystals within the organic bone matrix (Boskey and Posner, 1984). The location and orientation of individual crystals is not random, but rather is guided by the chemistry and structure of collagen and noncollagenous proteins and small proteoglycans initiating and regulating crystal nucleation and growth between and within collagen fibrils (George and Veis, 2008). Within the collagen fibril the mineral is formed in-between the assembled collagen molecules (intrafibrillar mineralization) (George and Veis, 2008). Interfibrillar crystals can be nucleated by the SIBLING proteins bone sialoprotein and DMP1 (Hunter and Goldberg, 1993; Hunter et al., 1996; He et al., 2003). We assumed that nucleation centers (N) are required to initiate mineral precipitation (Hunter and Goldberg, 1993; He et al., 2003), and that nucleators appear during matrix maturation. The number of nucleators per mature collagen molecule is k_2. We assumed that intrafibrillar and interfibrillar nucleators act in a similar way, therefore when $k_2 = 1$, there is only one intrafibrillar nucleator per 1 molecule of collagen, and when $k_2 > 1$, then there is a mix of intrafibrillar and interfibrillar nucleators. A resulting rate of nucleator appearance is proportional to matrix maturation given by Equation (1b) and is described by the term $k_2 \frac{dx_2}{dt}$ in Equation (1d). We assume that after mineralization is initiated by a given nucleator, this nucleator becomes a mineral crystal and thus can maintain, but no longer can initiate mineral precipitation (Hunter et al., 1996). Therefore, when mineraization starts, the number of nucleators decreases as they become masked by the mineral. The rate of decrease of nucleators was assumed to be proportional to the rate at which mineralized crystals (y) appear (dy/dt), as well as to the concentration of nucleators present, as described by the term $r_2 \frac{dy}{dt} N$ in Equation (1d).
4. The formation of mineral (y) was assumed to occur with a characteristic rate of k_3 and to be directly proportional to the number of nucleators and inversely related to the amount of inhibitors (Murshed and McKee, 2010). Although we modeled matrix mineralization in a homogenous assumption, it would be possible to relate the number of nucleators N to the number of mineral crystals within a given volume of the matrix, while the mineral growth rate k_3 to the growth of individual crystals. Particular considerations were given to the function describing the effect of inhibitors on mineral formation. We modeled mineralization rate by an equation of the form $\frac{dy}{dt} = k_3 g(I) N$, where $g(I)$ is a decreasing function of I which tends to 0 as I goes to infinity. Mineralization dynamics was qualitatively similar when $g(I)$ was described by the piecewise function $g(I) = \begin{cases} -aI + b, x \leq 1 \\ 0, I > 1 \end{cases}$ or the Hill type functions $g(I) = \frac{b}{b+e^{aI}}$ and $g(I) = \frac{b}{b+I^a}$, (data not shown). We chose a differentiable function amenable for biological interpretation $g(I) = \frac{b}{b+I^a}$ with $a = 10$ and $b = 0.001$. This function approaches 1 at I smaller than ~0.4, which represents the critical (nondimensionalized) value of I permitting mineralization in the system.

Based on these assumptions (**Figure 1**), the changes in the five components of the mineralizing bone matrix (**Table 1**) are described by the following system of ordinary differential Equations (1).

$$\frac{dx_1}{dt} = -k_1 x_1 \tag{1a}$$

$$\frac{dx_2}{dt} = k_1 x_1 \tag{1b}$$

$$\frac{dI}{dt} = v_1 x_1 - r_1 x_2 I \tag{1c}$$

$$\frac{dN}{dt} = k_2 \frac{dx_2}{dt} - r_2 \frac{dy}{dt} N \tag{1d}$$

$$\frac{dy}{dt} = k_3 \left(\frac{b}{b + I^a} \right) N \tag{1e}$$

Estimation of Characteristic Values of the Variables and Parameters

To estimate collagen packing within 1 μm^3 of matrix, we assume that a single molecule of triple-helical collagen is 1.4 nm in diameter and 300 nm in length based on estimates in the literature (Gross et al., 1955; George and Veis, 2008). Collagen molecules form fibrils of 70–90 nm in diameters, thus a single fibril contains ~3000 molecules (Hodge and Schmitt, 1960; George and Veis, 2008). We assume that fibrils have a ~10 nm coating of noncollagenous proteins and small proteoglycans, thus the cross-section of collagen fibrils is represented by circles of 110 nm diameter. In a hexagonal pattern circles have ~0.9069 packing density (Steinhaus, 1999), and hence, $(0.9069 \times 10^6/(\pi \times 55^2) \sim 95.4$ fibrils fit in a cross-section of 1 μm^2, and ~3.3 molecules fit in the 1 μm lengthwise. Therefore, in 1 μm^3 of volume, there are $95.4 \times 3.3 \times 3000 = 9.4 \times 10^5$ molecules of collagen.

To estimate the number of hydroxyapatite molecules, we started with a density of fully mineralized bone of 2.0 g/cm^3 (Gong et al., 1964). Assuming that bone contains 70% mineral, the hydroxyapatite density is ~1.40 $g/cm^3 = 1.40 \times 10^{-12}$ $g/\mu m^3$. Given the molecular weight of the hydroxyapatite molecule $[Ca_{10}(PO_4)_6(OH)_2]$ of 1004 g/mol, 1004 g contains 6×10^{23} molecules of hydroxyapatite. Therefore, 1 μm^3 of mineralized matrix contains $\sim 0.8 \times 10^9$ molecules of hydroxyapatite.

The number of nucleators was first assumed to be of the same order of magnitude as the number of collagen molecules

($k_2 = 1$), and later we examined how changing the number of nucleators affects the outcome of the mineralization. It is difficult to estimate the number of inhibitors, since we pooled into this category factors that use different mechanisms to achieve a single function—inhibition of mineralization. These factors are much smaller in size than the collagen molecules; however, there is less physical space available for them, and therefore we assumed that the numbers of inhibitor molecules and collagen molecules are of the same order of magnitude.

The rate constant values were chosen based on the observation that two main phases are present during bone mineralization: a slow phase of matrix maturation and a relatively fast phase of matrix mineralization (Boskey and Posner, 1984; George and Veis, 2008; Murshed and McKee, 2010). To account for this dynamic, we assumed that the rates of matrix maturation and the related processes of inhibitor processing and nucleator production are slower than the rates of mineral precipitation and nucleator removal/reduction. Since collagen maturation takes place by ∼10–14 days (Boskey and Posner, 1984; George and Veis, 2008), the rate of collagen assembly k_1 was estimated as 0.1 day^{-1}. We assumed the rate of inhibitor delivery to be $v_1 = 0.1$ day^{-1} and the rate of inhibitor degradation to be $r_1 = 2 \times 10^{-7}$ day^{-1} mol^{-1}. We assumed that when nucleators are available, and no inhibitors are present, mineralization occurs with a faster rate than collagen assembly $k_3 = 1000$ day^{-1}. An order of magnitude for the rate of the nucleator use by mineralization was $r_2 = 1.5$–2×10^{-8} mol^{-1}. The parameter values for the simulation of normal mineralization are given in **Table 2**. Further details for model nondimensionalization and numerical analysis are given in Supplementary Material.

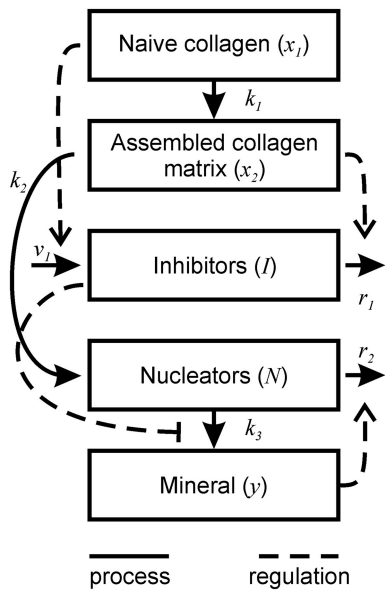

FIGURE 1 | Schematic representation of bone mineralization described by the model. Thick lines represent the processes occurring during mineralization. Dotted lines represent the regulatory effects of different components on the mineralization process.

Results

First, we examined the pattern of temporal changes in the five variables for the parameters representing bone mineralization in a healthy subject (**Tables 1, 2**). Naïve collagen, which initially constituted 100% of all collagen in the system, was gradually assembled into mature collagen, resulting in 80% conversion within 20 days, and in complete maturation within 40–60 days (**Figure 2A**). Inhibitors initially present in the naïve matrix were sustained for the first 10 days and rapidly degraded with the appearance of mature collagen (**Figure 2B**). Nucleating centers produced with the mature collagen reached the maximum at ∼10 days, and were removed with the offset of the mineralization (**Figure 2B**). After a lag time of ∼10 days, the mineralization first progressed rapidly followed by a continuous slow mineral formation (**Figure 2C**). The normalized mineralization degree of 1 (i.e., full mineralization) was reached ∼100 days after the deposition of naïve collagen. Thus, the model describes the lag time required for matrix maturation, the rapid mineralization offset, and the continuous slow increase in mineralization with time (Roschger et al., 2008b).

To model mineralization defects, we first examined the effect of the rate of hydroxyapatite formation k_3 on the mineralization outcome (**Figure 3**). Changes in k_3 predictably affected the rate of mineral formation, but also strongly and proportionally affected the degree of mineralization. A 3-fold decrease in the rate of hydroxyapatite formation k_3 resulted in a 3-fold decrease in mineralization degree (**Figures 3A,B**), while a 3-fold increase in k_3 led to a 3-fold increase in mineralization degree (**Figures 3C,D**). The robust effect of k_3 on the degree of mineralization is due to the fact that the removal of nucleators from the model is regulated by two independent parameters—the rate of hydroxyapatite formation (directly affected by k_3), and the efficiency of nucleator removal (r_2), which remains high even when k_3 is low. Therefore, when the rate of hydroxyapatite

TABLE 1 | Variables used in Equation (1).

Variables	Concentration represented	Characteristic values
x_1	Collagen matrix (molecules/μm^3)	9.4×10^5 molecules/μm^3
x_2	Assembled collagen matrix (molecules/μm^3)	9.4×10^5 molecules/μm^3
I	Inhibitor concentration (molecules/μm^3)	$\sim 10^6$ molecules/μm^3
N	Nucleator concentration (molecules/μm^3)	1–10 per 1 assembled collagen
y	Hydroxapatite (molecules/μm^3)	0.8×10^9 molecules/μm^3

TABLE 2 | Parameters used in Equations (1) and (2).

Parameter	Description	Value	Nondimensionalized
k_1	Collagen assembly	0.1 day^{-1}	0.1
k_2	Number of nucleators per collagen molecule	1	1
k_3	Formation of hydroxyapatite molecules	1000 day^{-1}	1
υ_1	Production of inhibitors by osteoblasts	0.1 day^{-1}	0.1
r_1	Degradation of inhibitors	2×10^{-7} day^{-1}	0.2
R_2	Use of nucleators by mineralized bone	1.7×10^{-8}mol^{-1}	12
a	Hill coefficient	10	10
b	Apparent dissociation constant for Hill function	10^{57}	0.001

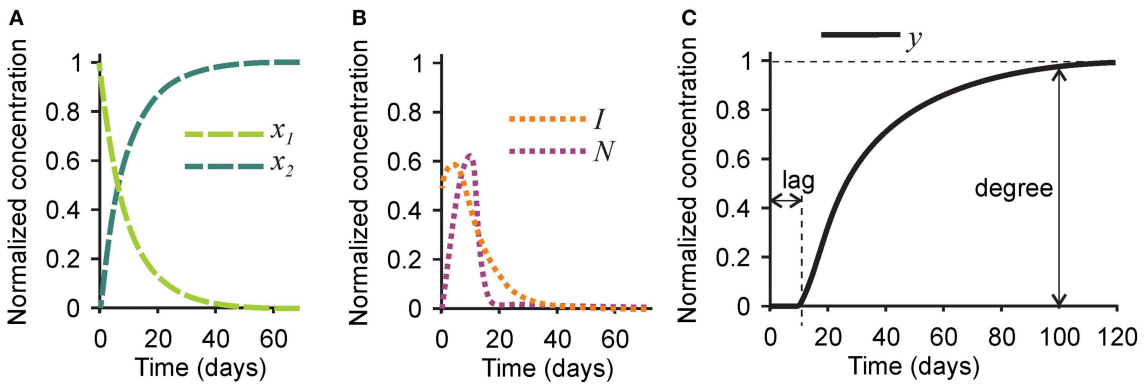

FIGURE 2 | Changes in time in different players in the mineralization process in healthy bone. (A) The concentrations of naïve (x_1, light green) and mature (x_2, dark green) collagen matrix. **(B)** The concentration of the mineralization inhibitor (I, orange) and the nucleation centers (N, purple). **(C)** The concentration of mineral (y). Indicated are the mineralization lag time, measured as a time delay between time 0 and the onset of mineralization, and mineralization degree, measured as the amount of mineral at time = 100 days. For the simulation of healthy bone, the mineralization lag time is 10 days, and the mineralization degree is 1.

formation decreases, the time interval during which nucleators are present remains unchanged, resulting in a decrease in mineralization degree. Changes in k_3 did not affect the dynamics of collagen maturation or turnover of its inhibitors.

Next we examined the role of parameters affecting the nucleators, the number of nucleators per mature collagen k_2, and rate of removal of nucleators caused by hydroxyapatite formation r_2 (**Figure 4**). Mineralization lag time was not affected by changes in k_2 and r_2, since the dynamics of collagen and inhibitors does not depend on these parameters (**Figures 4C,F**). A 3-fold decrease in the number of nucleators per mature collagen k_2 resulted in a 40% decrease in mineralization degree (**Figures 4A,C**), while a 3-fold increase in k_2 led to a 60% increase in the mineralization degree (**Figures 4B,C**). A 3-fold decrease in the rate of nucleator removal caused by hydroxyapatite formation r_2 resulted in an almost 2-fold increase in mineralization degree (**Figures 4D,F**), while a 3-fold increase in r_2 resulted in 40% decrease in mineralization degree (**Figures 4E,F**).

To examine the effect of parameters affecting the homeostasis of inhibitors on the mineralization outcome, we changed the rates of inhibitor production ν_1 and degradation r_1 (**Figure 5**). Since Equations (1a) and (1b) are not affected by these parameters, no change in the degree or timing of collagen maturation was evident following changes in ν_1 and r_1. The rate of inhibitor production ν_1 was changed 10-fold since smaller changes only resulted in slight differences in the mineralization. A 10-fold decrease in the rate of inhibitor production ν_1 resulted in a ∼20% decrease in mineralization lag time and a similar 20% increase in mineralization degree (**Figures 5A,C**). A 10-fold increase in the rate of inhibitor production ν_1 led to a 3-fold increase in mineralization lag time and a 40% decrease in mineralization degree (**Figures 5B,C**). The effect of changing the rate of inhibitor degradation r_1 on mineralization mirrored the effects of changing the rate of inhibitor production ν_1, however, smaller, 3-fold alterations of r_1 were required to obtain noticeable effects on mineralization. A 3-fold decrease in r_1 resulted in a sustained inhibitor presence, a 2-fold increase in mineralization lag time and 40% decrease in mineralization degree (**Figures 5D,F**). A 3-fold increase in the rate of inhibitor degradation r_1 resulted in a 2-fold decrease in the mineralization lag time and 20% increase in mineralization degree (**Figures 5E,F**).

Finally, we examined the effect of changing the parameters affecting initial collagen density $x_1(0)$ and maturation k_1 on the mineralization outcome (**Figure 6**). Change in the initial density of naïve collagen $x_1(0)$ represents an altered ability of osteoblasts to produce collagen, or altered collagen packing. A 3-fold decrease in $x_1(0)$ resulted in a proportionally lower amount of mature collagen and the number

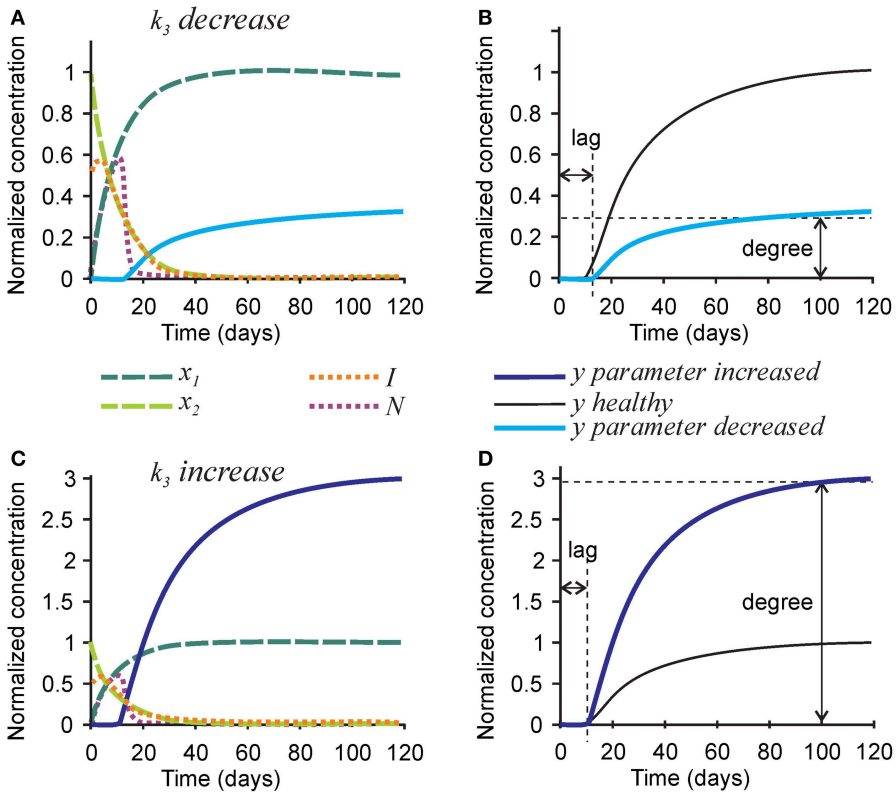

FIGURE 3 | The effect of parameter affecting formation of hydroxyapatite crystals k_3 on the mineralization outcome. (A) The effect of decreasing k_3 3-fold. **(B)** Comparison of the mineralization lag time and degree following decrease in k_3 to healthy mineralization. **(C)** The effect of increasing k_3 3-fold. **(D)** Comparison of the mineralization lag time and degree following increase in k_3 to healthy mineralization. The same color scheme is used as in **Figure 2**.

of nucleators, leading to a 2-fold decrease in mineralization degree (**Figures 6A,C**). In addition, the inhibitor presence was sustained for a longer period of time leading to a 2-fold increase in mineralization lag time (**Figure 6A**). A 3-fold increase in $x_1(0)$ led to a 3-fold increase in the amount of mature collagen and in the number of nucleators, which however translated to only a 70-80% increase in mineralization degree (**Figures 6B,C**).

A 3-fold decrease in the rate of collagen maturation k_1 resulted in the persistence of naïve collagen for up to 100 days and sustained inhibitor presence, leading to an almost 3-fold increase in mineralization lag time (**Figure 6D**). After mineralization started, it proceeded slower in the initial phase than in control conditions (**Figures 6D,F**). However, slow delivery of nucleators into the system resulted in a decrease in the rate of their removal (when nucleators are present at a low density, each of them can participate in mineralization for a longer time since they interfere less with each other). As a result, the mineralization rate did not decrease with time and a notably increased mineralization degree was reached (**Figures 6D,F**). A 3-fold increase in the rate of collagen maturation resulted in faster elimination of inhibitors and a slightly decreased mineralization lag time. The initial mineralization proceeded faster; however, because of faster removal of nucleators, it leveled off at lower overall mineralization degree (**Figures 6E,F**).

Discussion

The mathematical model for bone mineralization developed in this study captures the strongly nonlinear dynamics of mineralization, which starts from a lag phase when osteoid is present but no mineralization is evident, followed by fast primary mineralization, and subsequent secondary mineralization characterized by a continuous slow increase in bone mineral content (Roschger et al., 2008b). This dynamic was achieved in the model by assuming that (*i*) mineralization is suppressed in the presence of inhibitors, (*ii*) mineralization occurs fast, but requires the presence of nucleators, and (*iii*) nucleators formed during collagen maturation are removed from the system proportionally to the rate of mineralization. As a result, the lag phase allows for accumulation of nucleators, so that when inhibitors are reduced a large number of nucleators are present allowing mineralization to proceed rapidly. However, fast mineralization causes fast removal of nucleators leading to a substantial decrease in mineralization rate with time. We examined how changes in different parameters affect mineralization dynamics. The parameters describing the

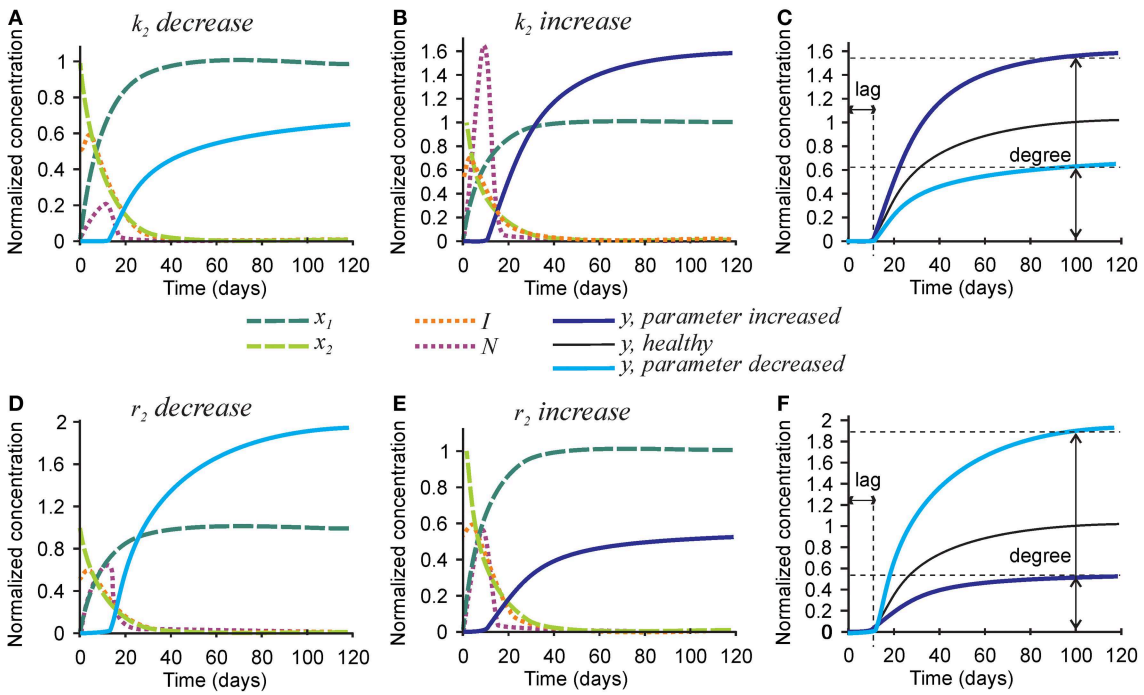

FIGURE 4 | The effect of parameters affecting nucleator production and removal on the mineralization outcome. (A–C) The effect of decreasing 3-fold **(A)** or increasing 3-fold **(B)** the number of nucleators per crosslinked collagen (k_2). **(C)** Comparison of the mineralization lag time and degree in conditions affecting k_2 to healthy mineralization. **(D–F)** The effect of decreasing 3-fold **(D)** or increasing 3-fold **(E)** the rate of use of nucleators by mineralized bone (r_2). **(F)** Comparison of the mineralization lag time and degree in conditions affecting r_2 to healthy mineralization. The same color scheme is used as in **Figure 2**.

formation of hydroxyapatite crystals at the nucleating centers potently affected the degree of mineralization, while the parameters describing inhibitor homeostasis effectively changed the mineralization lag time. Of interest, a single parameter describing the rate of matrix maturation was capable of counter-intuitively increasing both the mineralization lag time and the degree of mineralization.

The model represents an intentional simplification of a complex mineralization process, as we focused on simultaneously capturing mineralization-related functions of many regulatory molecules. Therefore, the following limitations should be noted: (1) The model does not specify different steps of matrix maturation, such as post-translational modification of collagen and noncollagenous matrix proteins, and collagen crosslinking. (2) The action of a large number of chemically distinct inhibitors is pooled together as a single entity. (3) Similarly, the difference in action of intrafibrillar and interfibrillar nucleators is not described. (4) The model does not contain the physical limitation for the maximal amount of mineral that can be deposited into the matrix, and therefore its long-term predictions should be interpreted with caution. Model applicability at this stage is limited to situations when the changes in mineralization dynamics are dramatic, while further development of the model is required to predict more subtle changes over time such as occurring during development and in complex disorders of osteoporosis and diabetes.

To compare the model predictions to the phenotype of bone disorders known to result in abnormal mineralization, we examined hypomineralization in osteomalacia and hypermineralization in osteogenesis imperfecta. We used the proportion of osteoid (osteoid volume per bone volume OV/BV, and/or osteoid thickness O.Th.) as an indicator of mineralization lag time, and bone mineral density distribution (BMDD) (Roschger et al., 2008b) as a measure of mineralization degree to relate the disease mineralization phenotype observed on histomorphometric and BMDD analysis to model predictions.

Application of the Model to Osteomalacia

Osteomalacia arises in part because of a systemic deficiency in calcium and/or phosphate ions and the hormones responsible for their regulation—vitamin D and FGF23. It is characterized by an increase in mineralization lag time and a decrease in mineralization degree (Arnala et al., 2001; Roschger et al., 2003; Rabelink et al., 2011; Cheung et al., 2013). It is assumed that the main cause of osteomalacia is a decreased rate of hydroxyapatite formation (reflected by the parameter k_3 in the model) caused by a low level of calcium and/or phosphate. However, the model predicts that a decrease in k_3 accounts only for a strong decrease in mineralization degree, but cannot by itself affect the mineralization lag time (as mineral formation starts only after the lag phase is completed). In order to account for the strong increase

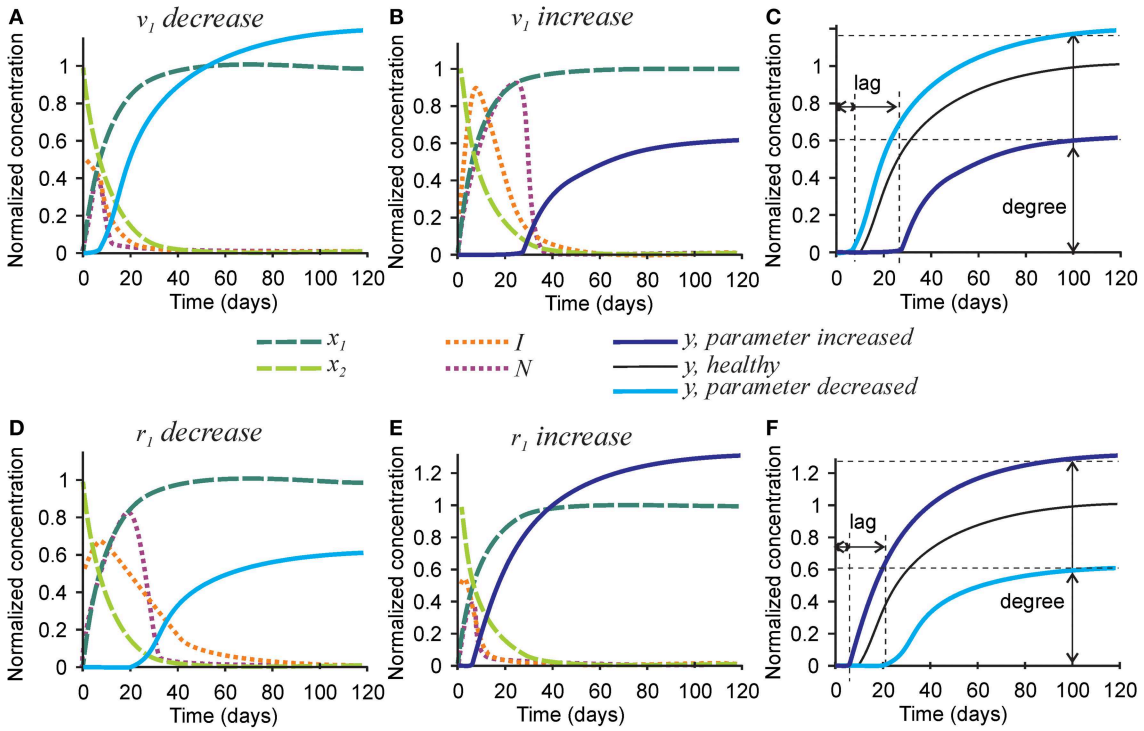

FIGURE 5 | The effect of parameters affecting inhibitor production and degradation on the mineralization outcome. **(A–C)** The effect of decreasing 10-fold **(A)** or increasing 10-fold **(B)** the rate of inhibitor production (v_1). **(C)** Comparison of the mineralization lag time and degree in conditions affecting v_1 to healthy mineralization. **(D–F)** The effect of decreasing 3-fold **(D)** or increasing 3-fold **(E)** the rate of inhibitor degradation (r_1). **(F)** Comparison of the mineralization lag and degree in conditions affecting r_1 to healthy mineralization. The same color scheme is used as in **Figure 2**.

in the mineralization lag time, it is necessary to assume additional direct or indirect effects of calcium/phosphate deficiency on inhibitor homeostasis. In fact, increases in local extracellular matrix mineralization inhibitors were also shown to contribute to the development of osteomalacia (Harmey et al., 2006; Barros et al., 2013; Millán, 2013). Inorganic calcium and phosphate are known to affect osteoblast differentiation (Beck et al., 2003; Dvorak et al., 2004), which could in turn result in changes in expression and processing of mineralization inhibitors. Deficiency in active 1,25-dihydroxyvitamin D (1,25(OH)2D) often associated with rickets (Takeda et al., 1997; Fukumoto, 2014) can affect the vitamin D receptor-mediated expression of mineralization inhibitors such as DMP1 (Nociti et al., 2014). Moreover, degradation of a strong inhibitor of mineralization—pyrophosphate (Addison et al., 2007)—is regulated by the concentration of phosphate, and both phosphate and pyrophosphate regulate expression of osteopontin (Harmey et al., 2006; Addison et al., 2007). In this context, removal/reduction of inhibitory osteopontin and its inhibitory peptides can be achieved by their extensive degradation by the enzyme PHEX (Addison et al., 2010; Barros et al., 2013). The model suggests that alteration of local inhibitor homeostasis is as important for the development of osteomalacia as is the direct effect of low calcium and phosphate on the rate of hydroxyapatite formation.

Application of the Model to Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a disease characterized by high bone fragility associated with low bone mass as well as high mineral content in the bone tissue resulting in its brittleness (Roschger et al., 2008a). Mutations in genes coding for collagen type I—the usual cause of osteogenesis imperfecta—are associated with hypermineralization and normal mineralization lag time (Rauch et al., 2000). In the model, an increase in the number of nucleators per molecule of collagen (k_2) results in an increase in mineralization degree but does not affect the mineralization lag time. Therefore, the model suggests that the hypermineralization in OI caused by mutations in type I collagen-encoding genes is attributable to the increase in the number of nucleators per molecule of collagen. This prediction is consistent with a recent study that demonstrated that the hydroxyapatite crystal size is similar in OI and control bone tissue, thus implying that the increased mineral content in OI must be due to an increased density of mineral crystals (Fratzl-Zelman et al., 2014). Since in the model the density of nucleating centers corresponds to the density of mineral crystals, we conclude that the model correctly predicts hypermineralization in OI due to mutations in genes coding for collagen type I.

Of interest, there are two distinct forms of OI in which different mineralization phenotypes are described. In OI

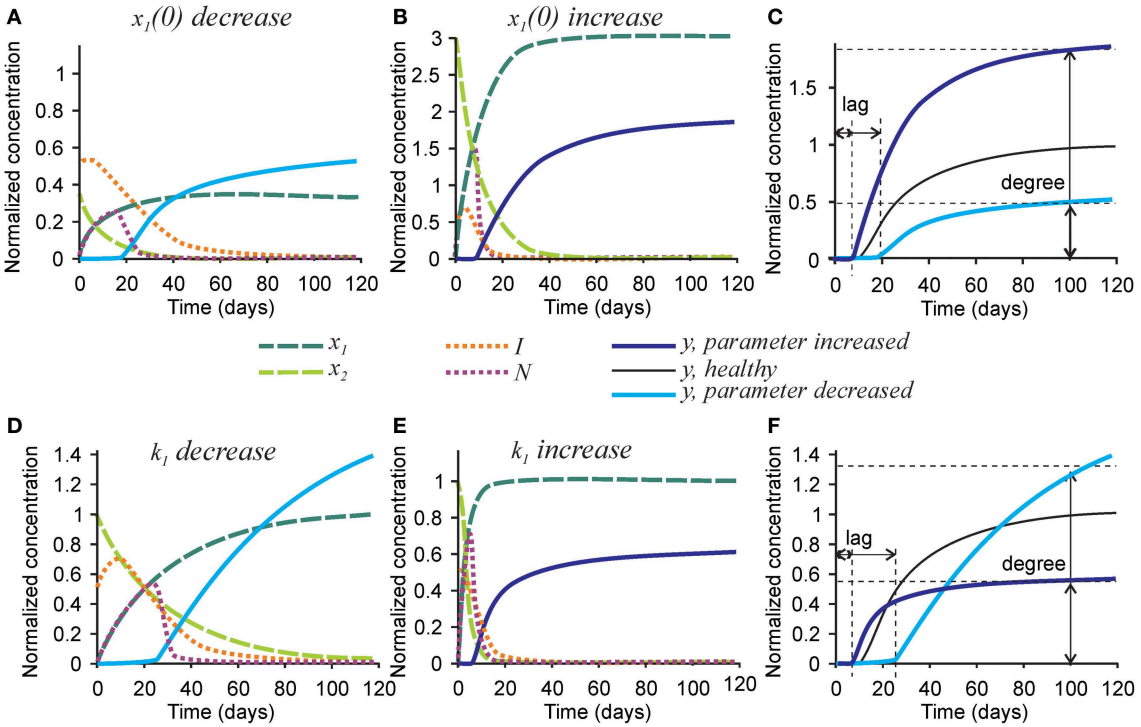

FIGURE 6 | The effect of parameters affecting collagen maturation on the mineralization outcome. (A–C) The effect of decreasing 3-fold **(A)** or increasing 3-fold **(B)** the amount of naïve collagen deposited by osteoblasts at time = 0 ($x_1(0)$). **(C)** Comparison of the mineralization lag time and degree in conditions affecting $x_1(0)$ to healthy mineralization. **(D–F)** The effect of decreasing 3-fold **(D)** or increasing 3-fold **(E)** the rate of collagen maturation (k_1). **(F)** Comparison of the mineralization lag and degree in conditions affecting k_1 to healthy mineralization. The same color scheme is used as in **Figure 2**.

caused by mutations in cartilage-associated protein (CRTAP) a significant increase in the mineralization degree (Fratzl-Zelman et al., 2010) and a marked reduction in mineralization lag time (Morello et al., 2006) were observed. In contrast, mutations in the collagen type I C-propeptide cleavage site give rise to a hypermineralization accompanied by a simultaneous increase in the mineralization lag time (Lindahl et al., 2011).

CRTAP forms a complex with P3H1 and cyclophilin B which 3-hydroxylates the Pro986 residue of collagen alpha chains (Chang et al., 2010). It was reported that CRTAP deficiency results the deposition of abnormally structured collagen fibrils (variable in diameter, with irregular borders) in skin samples of OI patients due to CRTAP mutation (Valli et al., 2012). The model predicts that an increase in the initial collagen density ($x_1(0)$) can result in hypermineralization accompanied by a significant decrease in mineralization lag time. It is important to stress that the model describes the changes occurring in the already-deposited collagen, but not the rate of its deposition by osteoblasts, which is negatively affected by CTRAP mutation. It is indeed noticeable, that the distance between the collagen fibers in the skin of a patient with CRTAP mutation appear to be smaller (Valli et al., 2012). Thus, the CRTAP mutation likely affects the packing of collagen molecules simultaneously resulting in (*i*) an increase in trapping/masking and degradation of inhibitors, thus shortening the mineralization lag time, and (*ii*) an increase in the density of nucleators leading to an increase in mineralization degree.

Mutation in the collagen C-propeptide cleavage site disrupts extracellular collagen processing, resulting in decreased collagen maturation rate (Lindahl et al., 2011), represented in our model by the parameter k_1. In the model, decrease in k_1 uniquely gave rise to the phenotype of increase in both mineralization lag time and degree. Conversely, mutation in *BMP1*—an enzyme that cleaves C-propeptide off procollagen—also results in a decrease in collagen maturation, hyperosteoidosis and hypermineralization (Hoyer-Kuhn et al., 2013). Thus, our model predicted a correct, albeit counter-intuitive, mineralization phenotype resulting from a decrease in the collagen matrix maturation rate.

Conclusion

We have developed a simplified mathematical model that describes changes in the mineralization of bone matrix when individual processes occurring during mineralization are altered. We validated the accuracy of model predictions using bone diseases associated with dramatic changes in mineralization

dynamics. During model development we used the data relevant to the mineralization process in human bone and applied the model to the analysis of human disorders of bone mineralization. In the future, this model can be applied for qualitative predictions of genotype/phenotype relationship in mouse models of bone mineralization, and it can be adapted to study mineralization of other calcified tissues, such as tooth dentin, cementum and enamel.

Author Contributions

SK, MDM, MM, and FR developed a biological framework of the model; LS, JG, EZ, MO, and SK constructed the model; LS, JG, EZ analyzed the model and generated the figures; SK prepared the first draft, all the co-authors read, critically revised, and approved the final manuscript.

Acknowledgments

The study was initiated and most of the work was completed during the Fifth Montreal Problem-Solving Workshop, August, 2013, which was supported by Network of Centre of Excellence Mprime, Group for Research in Decision Analysis (GERAD), the Matrix Dynamics Group, and Réseau de Recherche en santé Buccodentaire et Osseuse (RSBO). The completion of the project was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant number RGPIN-2015-05579 to SVK.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2015.00051

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 22 December 2015
doi: 10.3389/fcell.2015.00081

The Good and Bad of β-Catenin in Kidney Development and Renal Dysplasia

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Congenital renal malformations are a major cause of childhood and adult onset chronic kidney disease. Identifying the etiology of these renal defects is often challenging since disruptions in the processes that drive kidney development can result from disruptions in environmental, genetic, or epigenetic cues. β-catenin is an intracellular molecule involved in cell adhesion, cell signaling, and regulation of gene transcription. It plays essential roles in kidney development and in the pathogenesis of renal dysplasia. Here, we review the function of β-catenin during kidney development and in the genesis of renal dysplasia.

Keywords: kidney development, beta catenin, renal dyspalsia, ureteric epithelium, metanephric mesenchyme, renal stroma

OPEN ACCESS

Edited by:
Sunder Sims-Lucas, University of Pittsburgh, USA

Reviewed by:
Sun-Young Ahn, Children's National Medical Center, USA
Jacqueline Ho, Children's Hospital of Pittsburgh of UPMC, USA

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 July 2015*
Accepted: *04 December 2015*
Published: *22 December 2015*

Citation:
Boivin FJ, Sarin S, Evans JC and Bridgewater D (2015) The Good and Bad of β-Catenin in Kidney Development and Renal Dysplasia. Front. Cell Dev. Biol. 3:81. doi: 10.3389/fcell.2015.00081

INTRODUCTION

Renal dysplasia is a developmental disorder of the kidney that affects 1 in 250 live births (N'Guessen et al., 1984; Pohl et al., 2002). This disorder can lead to childhood renal failure, adult onset chronic renal insufficiency, and hypertension. Currently there are no cures for this disease and treatment is limited to managing the symptoms. This is due, in part, to a lack of understanding of the mechanisms of the pathogenesis of renal dysplasia. During normal kidney development β-catenin is expressed in the ureteric epithelium, metanephric mesenchyme, and renal stroma. Within these cell populations β-catenin modulates genetic programs that are essential for the control of branching morphogenesis and nephrogenesis. In human dysplastic kidneys, β-catenin is overexpressed in the ureteric epithelium, metanephric mesenchyme, and renal stroma suggesting a pathogenic role in renal dysplasia (Hu et al., 2003; Sarin et al., 2014). By manipulating the levels of β-catenin in animal models, studies have provided significant insight into the normal and pathogenic roles for β-catenin in kidney formation and the genesis of renal dysplasia. Here we review the normal developmental functions of β-catenin in the different cell lineages of the kidney and the contribution of β-catenin to renal dysplasia.

ANATOMY OF MAMMALIAN KIDNEY DEVELOPMENT

Metanephric kidney development is dependent upon the interactions of three main cell types: the ureteric epithelium, the metanephric mesenchyme, and the renal stroma. The formation of the mammalian kidney begins at embryonic day (E) 9.5 in the mouse when epithelial cells emerge from the intermediate mesoderm (termed the Wolffian duct) and migrate caudally toward the urogenital sinus (**Figure 1A**). At E10.5 in the mouse and 5 weeks gestation in humans an outgrowth of the caudal portion of the Wolffian duct, termed the ureteric bud, migrates into the adjacent metanephric mesenchyme (**Figure 1A**). In response to signals from the metanephric mesenchyme, the ureteric epithelium undergoes the first branching event to form a T-shaped branch at E11.5 in

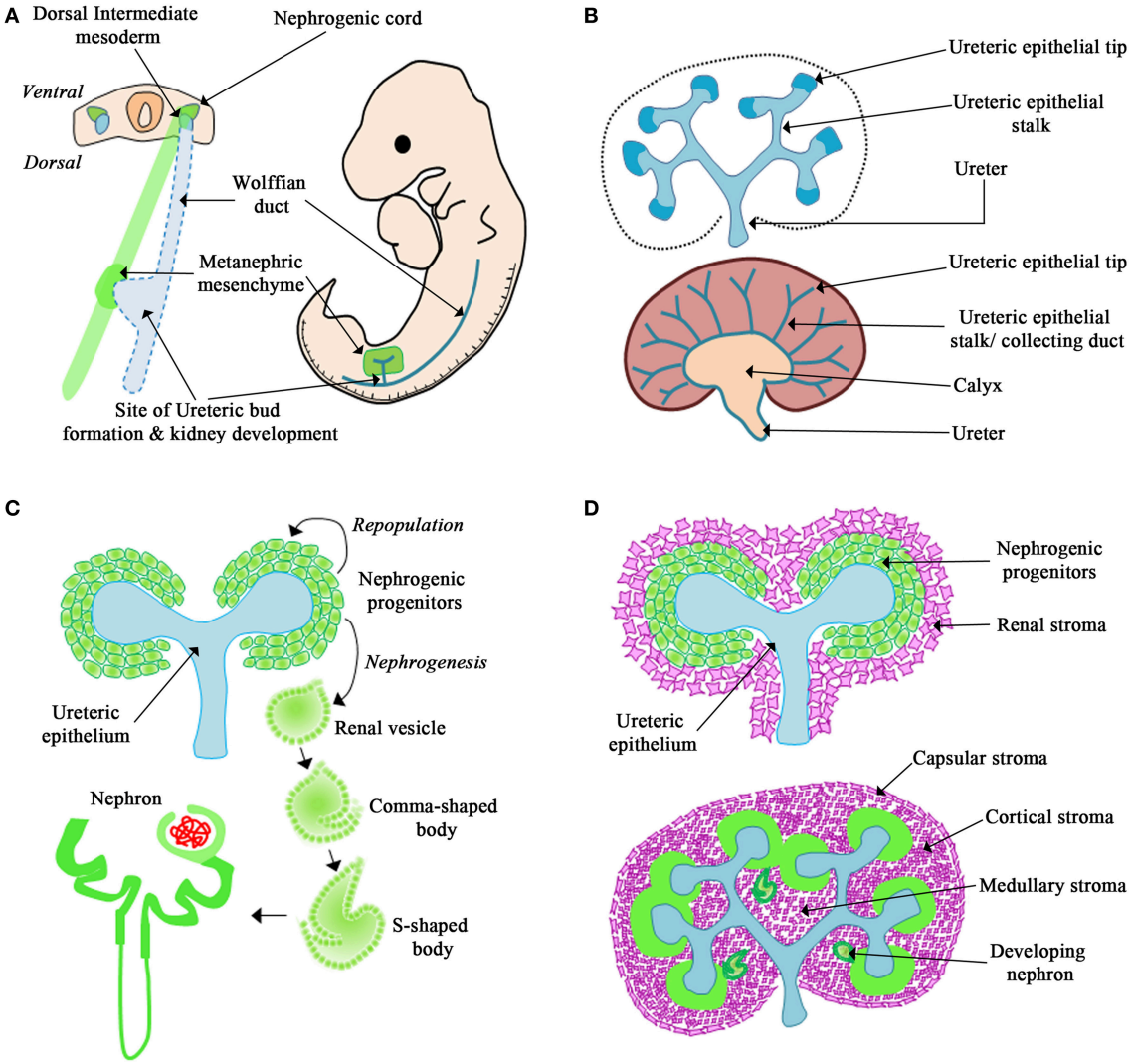

FIGURE 1 | Overview of kidney development. (A) The Wolffian duct originates from the dorsal intermediate mesoderm. The metanephric mesenchyme originates from the nephrogenic cord. The Wolffian duct forms an outgrowth called the ureteric bud that migrates into the surrounding mesenchyme and undergoes the first dichotomous branching morphogenesis event. **(B)** The ureteric epithelium is composed of the ureteric tip and stalk region. In response to signals from the mesenchyme, the tips of the ureteric epithelium undergo continued branching morphogenesis and the stalks will differentiate to form the collecting duct system, renal calyx, and ureter. **(C)** In response to signals from the ureteric epithelium the mesenchyme forms a cap around the ureteric tip. This cap mesenchyme will differentiate into nephrons or repopulate the nephrogenic progenitors. **(D)** The renal stroma surrounds the nephrogenic progenitors and will differentiate to form the capsular, cortical, and medullary stroma.

the mouse (**Figure 1A**; Saxén and Sariola, 1987). Each ureteric bud tip subsequently undergoes reiterative cycles of elongation, bifurcation, and differentiation to give rise to the metanephric collecting duct system, renal calyces, and ureter (**Figure 1B**). This process is known as branching morphogenesis and continues for 10 cycles to form ∼1500 collecting ducts in mice (Cebrián et al., 2004) and 15 cycles to form 60,000 collecting ducts in humans (Saxén and Sariola, 1987).

Simultaneously, the metanephric mesenchyme receives signals from the ureteric epithelium, and responds by tightly clustering around the ureteric bud tips. These clusters of condensed mesenchyme form the nephrogenic progenitors, which are populations of cells destined to form the nephron (**Figure 1C**). Depending on the molecular cues from the ureteric epithelium, the nephrogenic progenitors either undergo self-renewal to repopulate the condensed mesenchyme or differentiate into epithelial cells (Little and McMahon, 2012). The newly formed nephrogenic epithelial cells will then undergo nephrogenesis, the process to form the nephron via a series of distinct morphological changes (i.e., renal vesicle, comma-shaped, and s-shaped bodies) to form the nephron in a process termed nephrogenesis. This process will give rise to 10,000 nephrons (Cebrián et al., 2004) in the mouse and 200,000–1.8

million nephrons in humans (Saxén and Sariola, 1987; Hughson et al., 2003; Cebrián et al., 2004; Cain et al., 2010).

Shortly after the invasion of the ureteric bud into the metanephric mesenchyme a third cell population, termed the renal stroma, is observed surrounding the condensed mesenchyme (**Figure 1D**; Hatini et al., 1996; Cullen-McEwen et al., 2005; Li et al., 2014). The renal stroma is a population of matrix-producing fibroblast cells that surround adjacent nephrogenic structures and collecting ducts (Li et al., 2014). Studies have demonstrated that the renal stroma cell population is required for kidney development by modulating branching morphogenesis and nephrogenesis (Das et al., 2013; Hum et al., 2014; Li et al., 2014; Boivin et al., 2015). However, the mechanism of how the renal stroma regulates kidney development is only beginning to be understood.

The impairment of the processes that guide branching morphogenesis and nephrogenesis during kidney development can result in reduced nephron number and abnormal collecting duct formation, which can increase the risk of developing childhood renal failure or adult onset chronic renal insufficiency.

THE TRADITIONAL AND NON-TRADITIONAL ROLES OF β-CATENIN

β-catenin is an evolutionarily conserved multi-functional protein involved in various cellular activities depending on its intracellular localization (Heuberger and Birchmeier, 2010). At the cell membrane, β-catenin binds to the intracellular domain of E-cadherin to form adherens junctions between adjacent epithelial cells. Within these junctions β-catenin binds to α-catenin to bridge E-cadherin to the actin cytoskeleton, a process that is required for cell movements during morphogenesis (**Figure 2A**; Gumbiner, 2000, 2005). β-catenin is also involved in canonical Wnt signaling (Clevers and Nusse, 2012; **Figure 2B**). In this pathway, the absence of a Wnt signal results in the recruitment of cytoplasmic β-catenin by a destruction complex that is composed of axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (Gsk3β) and casein kinase 1α (Ck1α; Logan and Nusse, 2004). This destruction complex binds and phosphorylates β-catenin at serine/threonine residues leading to ubiquitination and proteasomal degradation of β-catenin (**Figure 2B**). In the presence of a Wnt signal, Wnt ligands bind to the Frizzled and LRP5/6 co-receptor complex, which results in the phosphorylation of disheveled (Dsh). Phosphorylated Dsh sequesters the destruction complex to the cell membrane and away from β-catenin. As a result, β-catenin is not phosphorylated by the destruction complex and accumulates in the cytoplasm. β-catenin can then translocate to the nucleus where it binds to the TCF/LEF family of DNA-bound co-transcriptional activators to regulate gene transcription (**Figure 2B**; Zeng et al., 2008; MacDonald et al., 2009). Activation of this canonical Wnt/β-catenin mediated signaling pathway regulates several genes involved in cell proliferation, cell fate specification, and differentiation (Logan and Nusse, 2004).

While traditionally involved in canonical Wnt signaling, β-catenin is also involved within other signaling pathways. In the study of Bmp signaling during kidney development and disease, β-catenin interacted with Smad1 and Smad4. Together these proteins form a transcriptional complex (Hu et al., 2003) that controls c-myc, which then regulates kidney epithelial tubule proliferation and differentiation (Hu and Rosenblum, 2005). The molecular interactions between β-catenin and Smad proteins downstream of the Bmp and Tgf-β signaling pathways also control the expression of genes, such as Xtwn and Msx2, which are involved in cell fate determination in other organ systems and may also be important in the kidney (Labbé et al., 2000; Hussein et al., 2003).

More recent studies have demonstrated that β-catenin can interact with members of the Hippo signaling pathway to control organ size (**Figure 2C**). In the developing heart, nuclear β-catenin forms a transcriptional complex with the Hippo signaling effector YAP to regulate cardiomyocyte proliferation (Heallen et al., 2011). Similarly, β-catenin also forms a molecular complex with YAP, YES1, and TBX5 to regulate proliferation in various β-catenin-driven human cancer cell lines (Rosenbluh et al., 2012). Specifically in the condensed mesenchyme of the kidney, deletion of the Hippo signaling effectors Taz and Yap results in reduced expression of β-catenin target genes, *Amph*, *Tafa5*, *Pla2g7*, and *Cited1,* thus decreasing the nephrogenic progenitor cell population (Das et al., 2013). This suggests that β-catenin and the Hippo signaling effectors interact to modulate the nephrogenic progenitor cell population. Furthermore, β-catenin forms a transcriptional complex with Yap, Yes1, and Tbx5 and binds to the promoter region of anti-apoptotic gene *bcl2l1* in the developing kidney (Boivin and Bridgewater, 2014). However, this novel transcriptional complex's biological outcomes in the specific cells of the developing kidney remain to be explored.

β-catenin is also involved in the Gdnf/Ret signaling pathway. Gdnf is expressed by the condensing mesenchyme cells, and its receptor, Ret, localizes to the ureteric epithelium. Activation of the Ret receptor initiates ureteric bud outgrowth from the nephric duct and is essential for continued branching morphogenesis. Recent studies demonstrate that β-catenin interacts with the Ret receptor during kidney development. Upon stimulation of the Ret receptor by Gdnf, β-catenin is released from the cytoplasmic tail of Ret and migrates to the nucleus of ureteric epithelial cells (Sarin et al., 2014; **Figure 2D**). It is likely that this activation of β-catenin via Gdnf/Ret signaling regulates unique genetic targets to control specific biological processes during normal kidney development and kidney disease. This concept is supported by studies in thyroid carcinomas in which Ret/β-catenin signaling regulates *cyclinD1*, *Egr1*, and *JunB* to promote cell proliferation (Gujral et al., 2008). Altogether, these studies demonstrate that β-catenin is a promiscuous molecule, interacting and communicating within numerous signaling pathways and transcription factors to modulate specific biological processes.

β-CATENIN IN NORMAL KIDNEY DEVELOPMENT

During kidney development, β-catenin demonstrates a specific spatial and temporal expression pattern. In the embryonic mouse and human kidney, β-catenin is expressed in the ureteric

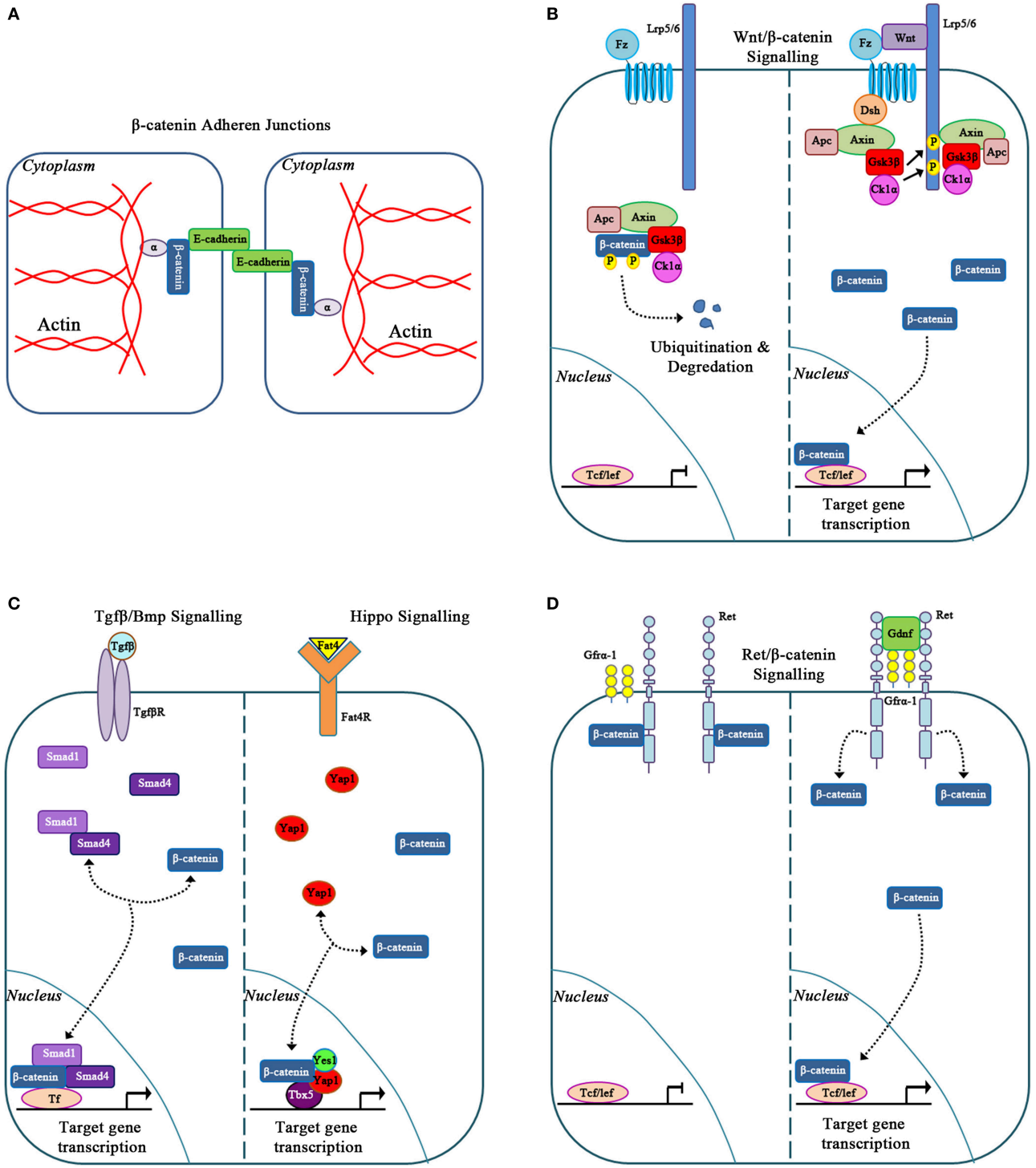

FIGURE 2 | Classic and novel functions for β-catenin. (A) β-catenin in cell to cell adhesion via adherens junctions. **(B)** β-catenin is a signaling molecule involved in Canonical Wnt signaling. **(C)** β-catenin forms novel molecular transcriptional complexes with Tgfβ/Bmp and Hippo signaling effectors. **(D)** β-catenin signals in response to Gdnf/Ret signaling.

epithelium, in all stages of the developing nephron, and in the renal stroma (Bridgewater et al., 2008; Sarin et al., 2014; Boivin et al., 2015). The cytoplasmic and nuclear expression patterns within these cells suggest functional roles in cell adhesion, cell signaling, and regulation of gene transcription. The analysis of transgenic mice containing *Tcf/Lef* binding sites upstream of a β-galactosidase reporter transgene confirmed β-catenin-mediated transcriptional activity in the Wolffian duct, ureteric epithelium, developing nephrons, and renal stroma (Iglesias et al., 2007; Bridgewater et al., 2008; Yu et al., 2009). Conditional mouse models have been used to greatly advance our understanding of the specific functional roles of β-catenin in the different cell lineages that are necessary for kidney development. These mouse models will be reviewed below.

β-Catenin in the Wolffian Duct and Ureteric Epithelium

One of the earliest known roles for β-catenin in kidney development is in the maintenance of *Gata3* expression within the Wolffian duct (Grote et al., 2008). *Gata3* controls proliferation and caudal extension of the Wolffian duct epithelium toward the urogenital sinus (Grote et al., 2006). *In silico* analysis of the Gata3 promoter region and β-galactosidase reporter assays demonstrated that Gata3 is a direct downstream transcriptional target of β-catenin (Grote et al., 2008). The targeted deletion of β-catenin from the Wolffian duct resulted in a loss of *Gata3* expression and failure to initiate normal ureteric budding, which ultimately leads to renal agenesis (Grote et al., 2008). In addition to β-catenin's effect on Wolffian duct elongation, β-catenin also plays essential roles in ureteric epithelial branching morphogenesis and differentiation (Bridgewater et al., 2008; Marose et al., 2008). Targeted inactivation of β-catenin in the ureteric lineage resulted in decreased expression of Emx2, a transcription factor essential in the maintenance of ureteric bud tip cells and branching morphogenesis (Miyamoto et al., 1997). This loss of Emx2 expression leads to reductions in its downstream targets *Lim1*, *c-Ret*, *Pax2*, and *Wnt11*, resulting in reduced branching morphogenesis and renal hypoplasia (Miyamoto et al., 1997; Bridgewater et al., 2008). The transcriptional control was likely direct in nature since the *Emx2* promoter contains numerous *Tcf/Lef* consensus binding sequences (Theil et al., 2002). Additionally, *Emx2* deficient mice exhibit branching defects and hypoplastic kidneys similar to the phenotype observed in mice deficient for β-catenin in ureteric epithelial cells (Miyamoto et al., 1997). Together, these studies support a role for β-catenin in regulating branching morphogenesis via the control of *Emx2* expression.

β-catenin is also required for proper ureteric epithelium differentiation. During the reiterative cycles of branching morphogenesis, the ureteric epithelium is spatially organized into the ureteric tip and stalk regions (**Figure 1B**). Each region displays distinct gene expression patterns (Bridgewater and Rosenblum, 2009) that are necessary for the development of the collecting duct system (Costantini, 2012). Marose et al. demonstrated that a loss of β-catenin from the ureteric epithelium leads to the activation of aquaporin-3 and ZO-1α+ in the ureteric bud tips. These genes are normally associated with the fully differentiated collecting duct system (Marose et al., 2008). Similarly, Bridgewater et al. demonstrated mice with a β-catenin deficiency in the ureteric epithelium exhibited a loss of ureteric bud tip associated genes. This led to a loss of tip cell identity and resulted in reduced branching morphogenesis (Bridgewater et al., 2008). Taken together, these studies demonstrate that β-catenin regulates a hierarchy of gene expression in the ureteric epithelium and maintains the ureteric bud tip identity, thus making the ureteric epithelium permissive for branching morphogenesis.

Considering the importance of β-catenin in cell adhesion and epithelial morphogenesis (Baum and Georgiou, 2011), two independent studies (Bridgewater et al., 2008; Marose et al., 2008) analyzed the effects of the loss of β-catenin in the ureteric epithelium on adherens junctions. These studies demonstrated that the formation and maintenance of adherens junctions were unaffected by the loss of β-catenin, likely due to the compensatory function of γ-catenin (Zhurinsky et al., 2000). These studies support the hypothesis that defects in branching morphogenesis result primarily from β-catenin mediated transcription (Bridgewater et al., 2008; Marose et al., 2008). However, the assembly and disassembly of adherens junctions is required for the complex movements of epithelial tubes during morphogenesis (Baum and Georgiou, 2011) and it would be interesting to determine if the dynamic nature of adherens junctions is maintained in the absence of β-catenin.

β-Catenin in the Mesenchyme

β-catenin plays essential roles in the induction of the condensed mesenchyme. This role was first demonstrated in *Wnt9b* deficient mice. During normal kidney development, Wnt9b, which is secreted from the ureteric epithelium, activates the canonical Wnt/β-catenin signaling pathway in the neighboring condensed mesenchyme cells. This inductive β-catenin mediated signal then up-regulates Wnt4 gene expression (Carroll et al., 2005; Park et al., 2007). Wnt4 activates downstream genetic targets *Fgf8*, *Pax8*, and *Lhx1* in a β-catenin dependent manner, which is essential for mesenchymal-to-epithelial transition (MET) and the formation of pre-tubular aggregates (Stark et al., 1994; Kispert et al., 1998; Park et al., 2007). The importance of β-catenin is also highlighted by the genetic deletion of β-catenin specifically in the nephrogenic progenitors. This resulted in reduced expression of early developing nephron markers *Fgf8*, *Pax8*, *Wnt4*, and *Lhx1* leading to stalled nephrogenic structures and markedly reduced nephrogenesis (Park et al., 2007). Furthermore, the sustained overexpression of β-catenin in the nephrogenic progenitors in *Wnt9b* deficient mice, which exhibit an absence of *Fgf8*, *Wnt4*, and *Lhx1,* resulted in the rescue of the expression of these nephrogenic genes (Park et al., 2007). This demonstrates β-catenin in the nephrogenic progenitors acts downstream of both Wnt9b and Wnt4 to initiate the nephrogenic program. While β-catenin signaling is sufficient to initiate nephrogenesis, it must be down-regulated for nephrogenesis to proceed to the renal vesicle stage (Park et al., 2007).

The deletion of β-catenin in the condensed mesenchyme results in the depletion of the nephrogenic progenitors thus

supporting a role for β-catenin in the maintenance or self-renewal of this cell population (Karner et al., 2011; Sarin et al., 2014). In the search for β-catenin targets involved in the maintenance of nephrogenic progenitors, Karner and colleagues demonstrated that β-catenin and Six2, a transcription factor essential for self-renewal of the nephrogenic progenitors (Kobayashi et al., 2008), work together to regulate the expression of genes in the nephrogenic progenitors (Karner et al., 2011). These candidate β-catenin target genes, among others, include *Cited1*, *Tafa5*, *Pla2g7*, and *Gdnf* (Karner et al., 2011). Their direct regulation by β-catenin is supported by co-immunoprecipitation and chromatin immunoprecipitation studies that demonstrate β-catenin forms a molecular complex with Six2 to bind to the promoter regions of nephrogenic progenitor genes, such as *Gdnf* (Park et al., 2012; Sarin et al., 2014). However, functional studies to determine if β-catenin and Six2 regulate the expression of all these genes have yet to be performed.

While investigating the cooperative role between Six2 and β-catenin, distinct gene expression domains within the condensed mesenchyme were identified. Park and colleagues demonstrated that Six2 and the Wnt9b/β-catenin signaling form gradients in the condensed mesenchyme to balance the proliferation and induction of the nephrogenic progenitors (Park et al., 2012). The condensed mesenchyme cells farthest from the ureteric epithelium receive a low Wnt9b signal, which results in low levels of β-catenin activity. Six2 is then able to repress β-catenin's transcriptional activity leading to self-renewal of the nephrogenic cell population. Conversely, the cells closest to the ureteric epithelium receive a high Wnt9b signal resulting in increased β-catenin activity. This leads to the initiation of nephrogenesis, activation of Wnt4, and MET (Park et al., 2012). This analysis demonstrates that the tight control of β-catenin activity is essential for balancing self-renewal of the nephrogenic progenitors and induction of nephrogenesis. It also suggests distinct zones within the nephrogenic progenitors that exhibit varying levels of β-catenin activity. Future studies are required to elucidate the specific β-catenin genetic targets and their functions within these distinct domains.

β-Catenin in the Renal Stroma

As kidney development progresses, the stromal population divides into three distinct populations: capsular, cortical, and medullary stroma. Within these three stromal populations the intracellular localization of β-catenin is variable, likely due to unique functional roles within each stromal cell sub-population (Boivin et al., 2015). In the renal capsule, β-catenin localizes primarily to the cell membrane. Since the integrity of the renal capsule is essential to maintain the high pressures within the kidney parenchyma, its primary role is likely involved in forming cell-cell junctions (Garcia-Estañ and Roman, 1989). Studies have also demonstrated that capsular stromal cells communicate with underlying cell populations, such as the cortical stroma and condensed mesenchyme (Levinson et al., 2005; Yallowitz et al., 2011). Mutant mice that lack β-catenin in stromal cells demonstrate a paucity of renal capsule with loosely packed capsular cells that are non-adherent to the underlying parenchyma (Boivin et al., 2015). These studies support a role for β-catenin in capsular cell-cell adhesion and capsular adhesion to the underlying cells. Further, these capsular defects likely contribute to disrupted communication with the cortical stroma and condensed mesenchyme. Thus, further investigation of β-catenin's role with respect to capsular stromal cell integrity is required to determine how it modulates cellular functions in the underlying cells.

The nuclear and cytoplasmic localization of β-catenin in the cortical stroma, which is directly adjacent to the condensed mesenchyme, suggests β-catenin may modulate cell signaling and gene expression to regulate nephrogenesis. In support of this theory, mutant mice with stromal β-catenin deficiency demonstrate a single layer of loosely packed condensed mesenchyme compared to *WT* kidneys, which exhibit 3–4 cell layers (Boivin et al., 2015). These alterations were caused by reduced levels of *Wnt9b* in the ureteric epithelium resulting in reduced expression of Wnt9b/β-catenin dependent genes (*Tafa5, Cited1,* and *Amph*) in the nephrogenic progenitor cells. These data suggest that β-catenin in the cortical stroma controls gene expression in neighboring cell populations possibly through direct cell-cell interactions or by β-catenin regulating secreted factors from the renal stroma. These possibilities are supported by studies that demonstrated renal stromal cells intermingle with the condensed mesenchymal cells and make direct contacts with the ureteric epithelium (Schnabel et al., 2003; Boivin et al., 2015). There is also evidence supporting stromal secreted factors signaling to the ureteric epithelium or mesenchyme to modulate gene transcription (Li et al., 2014). Future studies will be required to determine the identity of the factors controlled by β-catenin, the role of stromal cells interacting with ureteric epithelium, and the specific mechanisms of the stromal cells to modulate gene expression in the neighboring cells.

In medullary stromal cells, β-catenin primarily localizes to the nucleus (Boivin et al., 2015) and seems to play a prominent role in the regulation of gene transcription (Yu et al., 2009). The specific deletion of β-catenin in all stromal cells results in defects in cortico-medullary axis formation and a loss of medullary stroma (Yu et al., 2009). Yu et al propose that Wnt7b, secreted from the ureteric epithelium, signals to the medullary stroma and activates a β-catenin mediated signaling pathway that controls proper cortico-medullary patterning and contributes to epithelial tubule elongation (Yu et al., 2009; Maezawa et al., 2012; Boivin et al., 2015). While the specific genetic targets activated by Wnt7b/β-catenin signaling in the medullary stroma are not well defined, one possible target is the cyclin-dependent kinase inhibitor p57Kip2, which is markedly reduced in both *Wnt7b* and β-catenin stromal cell mutants (Yu et al., 2009). This suggests that β-catenin in the medullary stroma regulates genetic programs that control medullary stromal cell development and/or maintenance. Taken together, these studies highlight essential roles for β-catenin in the renal stroma.

β-CATENIN IN RENAL DYSPLASIA

Renal dysplasia is a congenital kidney malformation that affects up to 0.1% of live births and has an even higher prevalence of 4% in fetus and infants at autopsy (Chen and Chang, 2015). The

label "dysplasia" is given to kidneys with structural abnormalities that include, at the gross level, renal agenesis, hypoplasia, hypodysplasia, multiplex kidneys with duplicate ureters, and multicystic dysplastic kidneys (Woolf et al., 2004; Winyard and Chitty, 2008; Goodyer, 2009). At the histopathological level, dysplastic kidneys exhibit defects in cortical and medullary patterning (Piscione and Rosenblum, 1999), disorganization of the collecting system and nephron elements (Woolf et al., 2004), dilated/cystic epithelial tubules (Hu et al., 2003; Katabathina et al., 2010; Trnka et al., 2010), undifferentiated tubules and mesenchyme (Winyard and Chitty, 2008), cystic glomeruli (Sanna-Cherchi et al., 2007), and expanded loosely arranged stroma (Woolf et al., 2004; Winyard and Chitty, 2008). These abnormalities can be diffuse (involving the entire kidney), segmental (involving segments of the kidney) or focal (affected regions intermingled with normal tissue; Winyard and Chitty, 2008). Defects in the processes that guide kidney development are the major contributing factors leading to the gross and histopathological changes observed in renal dysplasia.

Despite our growing understanding of renal dysplasia, the precise causes and mechanisms contributing to the pathogenesis remain poorly understood. The abnormal expression of numerous growth factors, signaling pathways, and transcription factors has been observed in humans and mouse models of renal dysplasia (Jain et al., 2007; Bridgewater et al., 2011; Thomas et al., 2011). Furthermore, studies have linked specific mutations in genes such as Sal-like 1 (*Sall1*), Paired Box gene 2 (*Pax2*), and Transcription factor 2 (*TCF2*) to the genesis of renal dysplasia (Weber et al., 2006). The overexpression of β-catenin in both human renal dysplastic tissue with different underlying etiologies (Hu et al., 2003; Sarin et al., 2014) and in numerous mouse models of renal dysplasia (Bridgewater et al., 2011; Sarin et al., 2014), strongly suggest that overexpression of β-catenin is a contributing factor to the pathogenesis of renal dysplasia. The use of conditional mouse models and human dysplastic kidney tissue has greatly advanced our understanding of the pathogenic role of β-catenin overexpression (Hu et al., 2003; Sarin et al., 2014) and will be reviewed below.

β-Catenin Overexpression in the Ureteric Epithelium

Hu et al. demonstrated that β-catenin is overexpressed in ureteric epithelial derived structures in dysplastic human kidney tissue, and this was the first finding to highlight dysregulated β-catenin expression in human renal dysplasia (Hu et al., 2003). The functional role of β-catenin overexpression in the ureteric epithelium was initially investigated by creating a mouse model with targeted overexpression of β-catenin specifically in the ureteric epithelium (Bridgewater et al., 2011). These mutant mice exhibited severe renal hypodysplasia characterized by marked defects in branching morphogenesis and nephrogenesis (Bridgewater et al., 2011). In this transgenic model, a mechanism was defined in which β-catenin overexpression led to the upregulation of Tgfβ2 and the Wnt inhibitor *Dkk1* specifically in the ureteric epithelium. The overexpression of Tgfβ2 in epithelial cells inhibited ureteric branching and caused ectopic and premature differentiation of nephrogenic progenitors, while *Dkk1* inhibited Wnt4 activity (Bridgewater et al., 2011). In a similar study, Marose et al. demonstrated that ureteric-specific β-catenin overexpression in mice prevented ureteric epithelium differentiation to form collecting ducts resulting in hypodysplastic cystic kidneys (Marose et al., 2008). Together, these studies established that β-catenin overexpression results in marked alterations in branching morphogenesis and nephrogenesis due to disrupted gene expression in the ureteric epithelium.

β-Catenin Overexpression in the Metanephric Mesenchyme

In addition to β-catenin overexpression in ureteric epithelial cells, human dysplastic renal tissue also demonstrates β-catenin overexpression in the mesenchymal and stromal cells (Sarin et al., 2014). A close examination of the expression pattern showed that β-catenin was primarily increased in the mesenchymal and stromal cell nuclei suggesting a role in the regulation of gene transcription (Sarin et al., 2014). Park et al. demonstrated, using the *Six2-Cre* mice to overexpress β-catenin in the nephrogenic progenitors, an inability to form renal vesicles resulting in severely hypodysplastic kidneys (Park et al., 2007). Analysis of a mouse model using the *RARβ2Cre* (Kobayashi et al., 2005) to overexpress β-catenin in mesenchyme demonstrated histopathological characteristics consistent with human renal dysplasia, including the presence of large cortical and medullary cysts, undifferentiated mesenchyme, cystic glomeruli, expanded stromal cells surrounding dilated tubules, and multiplex kidneys containing numerous rudimentary kidney structures (sometimes up to eight per animal; Park et al., 2007; Sarin et al., 2014; **Figures 3C,D**). The multiplex kidneys observed in the mouse model resulted from β-catenin in the mesenchyme upregulating *Gdnf* expression and consequently the ectopic ureteric budding off the Wolffian duct (Sarin et al., 2014). This *Gdnf* overexpression was also observed in human fetal and postnatal dysplastic kidney tissue, specifically in the mesenchyme and on the surface of the ureteric epithelium. Interestingly, the analysis of human dysplastic tissue from the McMaster University Anatomy Program's pathology library revealed several cases of human renal dysplasia with multiplex kidneys, supporting the accuracy of the mouse model to the human condition (Sarin et al., 2014; **Figures 3A,B**). These studies demonstrate that mesenchymal overexpression of β-catenin disrupts branching morphogenesis and nephrogenesis by misregulating important kidney development genes in human and mouse models of renal dysplasia.

β-Catenin Overexpression in the Kidney Stroma

A notable histopathological feature of renal dysplasia is an expansion of the stroma cell population (Woolf et al., 2004; Winyard and Chitty, 2008). The analysis of β-catenin expression in human dysplastic kidneys revealed β-catenin is markedly increased, primarily in the nucleus of the expanded stromal population (Sarin et al., 2014). While the molecular mechanisms

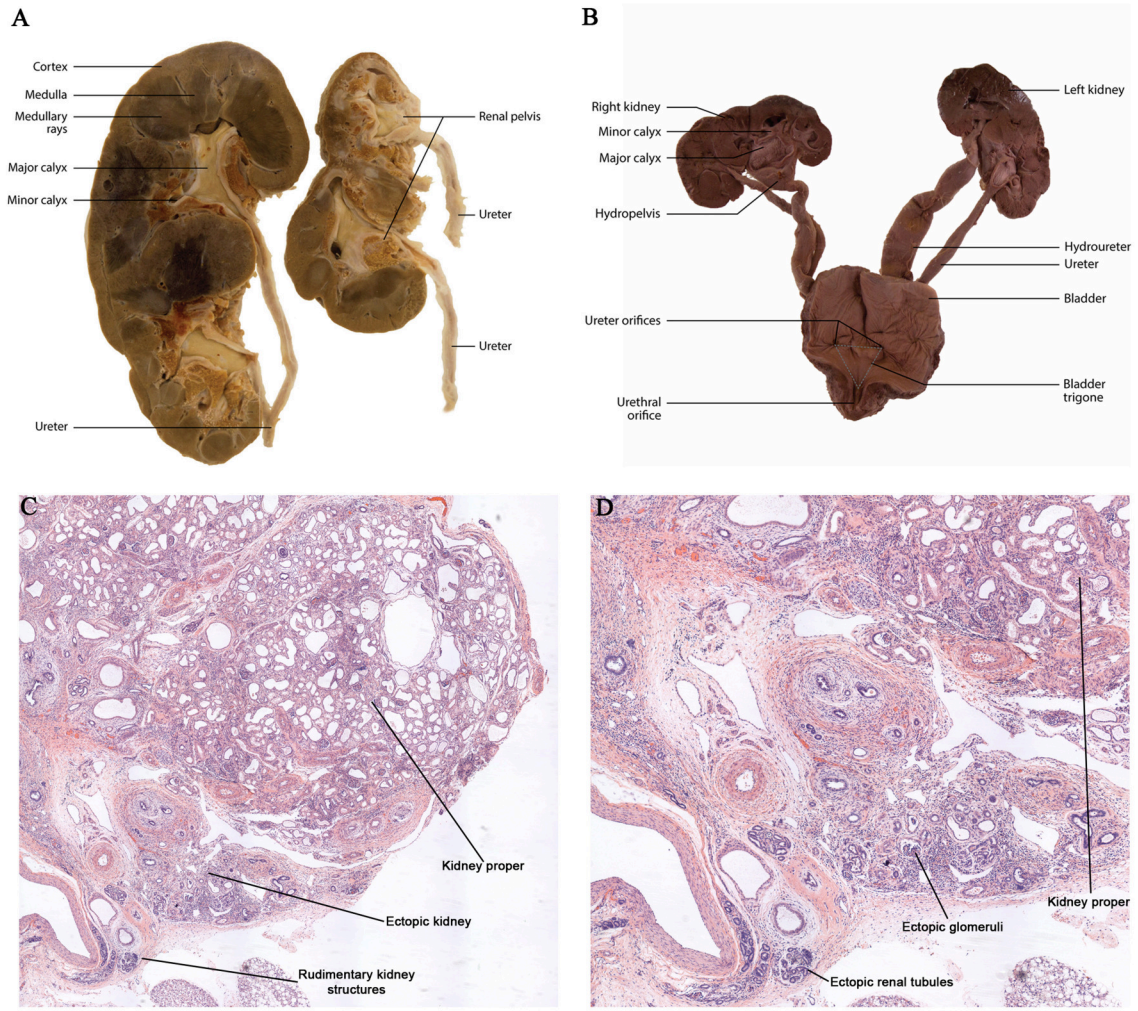

FIGURE 3 | Dysplastic multiplex kidneys. (A,B) Gross anatomy of post-natal and embryonic multiplex kidneys with bilateral bifid ureters. **(C,D)** Histological analysis of dysplastic kidneys demonstrating multiplex kidneys.

for an overexpression of stromally expressed β-catenin in the pathogenesis of renal dysplasia is not yet known, studies have provided some evidence to suggest a potential role (Maezawa et al., 2012; Sarin et al., 2014). In the embryonic mouse kidney the overexpression of β-catenin exclusively in stromal progenitors is sufficient to cause severe kidney developmental abnormalities. These include several characteristics consistent with renal dysplasia such as disrupted branching morphogenesis, reduced nephrogenesis, and an expansion of the stromal population (Boivin et al., 2015). Further evidence is provided in a mouse model that overexpresses β-catenin in both the condensed mesenchyme and stroma (Maezawa et al., 2012; Sarin et al., 2014). The kidney phenotype in these two models exhibited large, misshapen lobular dysplastic kidneys at birth (Maezawa et al., 2012; Sarin et al., 2014). Conversely, mice with β-catenin overexpressed exclusively in the nephrogenic progenitors result in renal agenesis at birth (Park et al., 2007). Therefore, the marked differences in the kidney phenotypes are likely due to the functional contributions of β-catenin in the renal stroma.

Congenital obstructive nephropathy includes myofibroblast transformation and interstitial fibrosis culminating in renal dysplasia (Nagata et al., 2002). The overexpression of β-catenin in postnatal medullary stroma was sufficient to promote interstitial fibrosis through an upregulation of α-smooth muscle actin and myofibroblast transformation (DiRocco et al., 2013). Considering β-catenin's role in postnatal fibrosis, it is likely that stromal β-catenin is also a central player in interstitial fibrosis observed in renal dysplasia caused by congenital obstructive nephropathy. Taken together, these studies highlight potential roles for β-catenin in the renal stroma in the pathogenesis of renal dysplasia.

CONCLUSION

Currently there are no cures for renal dysplasia and treatment is limited to managing the symptoms. The literature reveals β-catenin is a central player in kidney development and the pathogenesis of renal dysplasia. β-catenin is involved in regulating novel signaling pathways, transcriptional complexes, and genes that contribute to renal dysplasia. The continued

investigation of β-catenin in kidney development and disease will undoubtedly identify novel signaling pathways and transcriptional targets for preventative and therapeutic treatments.

FUNDING

This work was funded by NSERC (405644), Kidney Foundation of Canada, and CIHR (142264).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 11 November 2015
doi: 10.3389/fcell.2015.00070

Assembling Kidney Tissues from Cells: The Long Road from Organoids to Organs

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The field of regenerative medicine has witnessed significant advances that can pave the way to creating *de novo* organs. Organoids of brain, heart, intestine, liver, lung and also kidney have been developed by directed differentiation of pluripotent stem cells. While the success in producing tissue-specific units and organoids has been remarkable, the maintenance of an aggregation of such units *in vitro* is still a major challenge. While cell cultures are maintained by diffusion of oxygen and nutrients, three- dimensional *in vitro* organoids are generally limited in lifespan, size, and maturation due to the lack of a vascular system. Several groups have attempted to improve vascularization of organoids. Upon transplantation into a host, ramification of blood supply of host origin was observed within these organoids. Moreover, sustained circulation allows cells of an *in vitro* established renal organoid to mature and gain functionality in terms of absorption, secretion and filtration. Thus, the coordination of tissue differentiation and vascularization within developing organoids is an impending necessity to ensure survival, maturation, and functionality *in vitro* and tissue integration *in vivo*. In this review, we inquire how the foundation of circulation is laid down during the course of organogenesis, with special focus on the kidney. We will discuss whether nature offers a clue to assist the generation of a nephro-vascular unit that can attain functionality even prior to receiving external blood supply from a host. We revisit the steps that have been taken to induce nephrons and provide vascularity in lab grown tissues. We also discuss the possibilities offered by advancements in the field of vascular biology and developmental nephrology in order to achieve the long-term goal of producing transplantable kidneys *in vitro*.

Keywords: pluripotent stem cells, organoids, stem cell differentiation, kidney development, vascularization

OPEN ACCESS

Edited by:
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Reviewed by:
Kimberly Jean Reidy, Children's Hospital at Montefiore and Albert Einstein College of Medicine, USA
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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *14 August 2015*
Accepted: *23 October 2015*
Published: *11 November 2015*

Citation:
Hariharan K, Kurtz A and Schmidt-Ott KM (2015) Assembling Kidney Tissues from Cells: The Long Road from Organoids to Organs. Front. Cell Dev. Biol. 3:70. doi: 10.3389/fcell.2015.00070

INTRODUCTION

While our knowledge on the structure and function of mature organs is mostly derived from animal models, human cadavers, or biopsies, the information we have on embryonic organ development is largely derived from various model organisms and from technologies that study human embryogenesis *in vitro*. In 1944, the first human egg was fertilized *in vitro* (Rock and Menkin, 1944). Edwards and Steptoe implanted a fertilized egg into a woman's uterus in 1977 (Steptoe and Edwards, 1978) giving the world its first test tube baby. These scientists were

also paving the way for a closer observation of human embryonic development. Edwards and his team had worked on growing human embryos *in vitro*, until the blastocyst stage (Edwards et al., 1981) before re-implanting the embryo into the uterus. Knowing that *in vitro* culture was a suitable interim step in producing a viable organism spurred the goal of replacing damaged organs by transplanting organoids produced *in vitro*. Key advances in this field included the derivation of human embryonic stem cells (hESC) from the inner cell mass of normal human blastocysts (Thomson et al., 1998) and the generation of induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). These cells demonstrate evidence of stable developmental potential even after prolonged culture forming derivatives of all three embryonic germ layers from the progeny of a single cell. A huge advantage of iPSC over ESC, is the possibility to have pluripotent stem cells from every individual, providing an opportunity to study and repair genetic disorders at a personal level. Pluripotency has been exploited to recapitulate many embryonic processes *in vitro*, including primitive streak formation, neural tube induction, and trophoblast formation, extending to the generation of functioning neurons, beating cardio-myocytes, and insulin producing beta cells of the pancreas. Major achievements in the field of stem cell biology have been the derivation of a multitude of differentiated cell types morphologically similar to their terminally differentiated counterparts *in vivo*, but functionally immature *in vitro*, that have been intended to be used as regenerative cell sources in degenerative disease conditions. However, in order to model and study human disease, and to subsequently test and develop drugs, functionality of terminally differentiated cells has to be achieved.

Mammalian cells, as part of multicellular organisms, function in tissue units that contain several types of cells, which together form an organ. The fact that many ESC and iPSC—derived cell types display a mostly immature, fetal phenotype suggests that multicellularity is also a prerequisite for terminal differentiation. Nevertheless, the ability to derive specific cell types of any organ from PSCs has provided the possibility of constructing functional units of an organ, containing distinct cell types. Several groups pioneered the development of tissues *in vitro* from PSCs, including 3D cortical neuro-epithelium with up to 6 layers of neurons (Eiraku et al., 2008), intestinal (Spence et al., 2011), retinal, liver, inner ear, and kidney organoids (Eiraku et al., 2011; Nakano et al., 2012; Koehler et al., 2013; Takebe et al., 2013; Takasato et al., 2014) These novel findings prompted a redefinition of the term "organoid" as a collection of organ-specific cell types that develop from stem cells or organ progenitors and self-organize through cell sorting and spatially restricted lineage commitment in a manner similar to the situation *in vivo* (Lancaster and Knoblich, 2014). Despite all the progress, no functional nephron or liver acini unit has been generated *in vitro*. In this review, we will revisit methods to differentiate tissue-specific cells from pluripotent stem cells, to achieve 3-dimensional tissue modeling, and discuss current efforts to enhance vascularization of organoids *in vitro* and *in vivo*.

MIRRORING ORGANOGENESIS *IN VITRO*

Kidney-specific Cell Differentiation from Pluripotent Stem Cells

Humans are triploblastic and thus, develop a tri-laminar embryo upon gastrulation, consisting of ectoderm, endoderm, and mesoderm. Several groups have cultured PSCs in a feeder free matrix-coated surface and promoted differentiation by adding growth factors and inhibitors to this 2D system, while other groups applied inductive media to a 3D system, after the generation of a sphere of PSC cells known as an embryoid body (EB). Protocols applying defined time periods and concentrations of exposure to inductive agents have allowed for the differentiation of endo-, ecto-, or mesodermal lineages and specific cell types. For example, activin signaling leads to 80% efficient induction of $SOX17^+/GSC^+/FOXA2^+/MIXL1^+$ definitive endoderm in hESC cultures after 5 days of differentiation in 100 ng/ml activin A (D'Amour et al., 2005). In another seminal study, sequential treatment of high-density undifferentiated monolayer hESC cultures with activin A for 24 h and bone morphogenetic protein 4 (BMP4) for 4 days consistently yielded >30% cardiomyocytes (Laflamme et al., 2007). For neural induction of hESCs, the growth factors insulin, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were added in a chemically defined medium giving rise to up to 90% $PAX6^+$ neural progenitors (Joannides et al., 2007). These studies have proved that while a spontaneously differentiating mass of PSC can generate varying proportions of endo- and ecto- and mesoderm, targeting signaling pathways prominent in the development of an organ of interest in a temporal fashion can promote that particular lineage. Over the years, researchers have tested various combinations of cell signaling modifiers to achieve enrichment of tissue-specific cell types in a heterogenous population of differentiated PSC progeny (reviewed by Murry and Keller, 2008). Moreover, the level of enrichment varies with the iPSC or ESC used. iPSC that are reprogrammed from a somatic cell type originating from an organ and are differentiated to a cell type of the same organ, show higher efficiencies owing to epigenetic memory of the source (Hiler et al., 2015). This highlights the need for standardization of protocols with certain PSC lines for certain lineages.

Unlike other organs, few protocols have been established for the kidney. The kidney exhibits a remarkable architectural complexity coupled with the presence of at least 26 different specialized cells (Al-Awqati and Oliver, 2002). The kidney is mesodermal in origin, arising from the intermediate mesoderm (IM). Arising from IM, the ureteric bud (UB) interacts with an adjacent cell population, the metanephric mesenchyme (MM). Through reciprocal interactions, the ureteric bud will generate the ureteric tree giving rise to the ureter, renal pelvis and collecting ducts, while progenitors within the metanephric mesenchyme will generate nephrons (Grobstein, 1953; Saxén and Sariola, 1987). Based on earlier studies that highlighted important molecules and pathways that drive mesodermal and nephron differentiation in model organisms, initial studies of differentiation toward the renal lineage were performed on mouse ESC EBs treated with media containing serum together

with multiple combinations of factors including activin A, BMP4, BMP7, retinoic acid (RA), leukemia inhibiting factor (LIF), and glial-derived neurotrophic factor (GDNF) or UB-derived conditioned media. These protocols led to the generation of cells expressing markers of differentiation, e.g., Pax2 (kidney tubules), Aquaporin-2 (collecting duct principal cells), Wt1 (metanephric mesenchyme and podocytes), or Ksp-Cadherin (distal nephron tubules), within EBs, which provided evidence of successful renal lineage induction (Kobayashi et al., 2005; Bruce et al., 2007; Vigneau et al., 2007; Morizane et al., 2009; Ren et al., 2010; Nishikawa et al., 2012). Although a renal identity was achieved in the examples mentioned before, the desired cell types could not be isolated owing to low and varying frequencies of occurrence and their functionality was not demonstrated.

Meanwhile, genetic lineage tracing demonstrated that the induced Six2-expressing cap mesenchyme represents a nephron progenitor population that gives rise to all cell types of the nephron (Kobayashi et al., 2008). Also, the intermediate mesodermal origin of kidney cells was confirmed when Mugford et al., used molecular fate mapping to demonstrate that the majority of cell types within the metanephric kidney arise from an Osr1-expressing population within the intermediate mesoderm (Mugford et al., 2008). These new findings were considered in differentiation protocols to distinguish the exact mesodermal cell sub-type required to enrich cultures of PSC-derived renal progeny. For instance, Mae et al developed a robust protocol using activin A and Wnt-agonist CHIR99021 for 2 days and sequential treatment with BMP7 and CHIR99021 for 8 days to obtain 90% $OSR1^+$ cells (Mae et al., 2013). Despite such an efficient protocol, the dependence on OSR1 as a population identifier created ambiguity, since even though OSR1 is expressed in the intermediate mesoderm, it is also expressed in the earlier mesoderm prior its subdivision into paraxial and intermediate domains (Guillaume et al., 2009). Moreover, OSR1 is expressed in both the intermediate mesoderm and lateral plate (James and Schultheiss, 2003; Wilm et al., 2004).

Additional protocols were developed to induce different cell types within the nephron. Human PSC derived podocytes expressing podocin, nephrin, and synaptopodin were generated from EBs using treatment with Activin A, RA, and BMP7 and plating on gelatin (Song et al., 2012). In another study, ∼30% $AQP1^+$ proximal tubule cells were obtained by treating a monolayer PSC culture with media containing RA, BMP2, and BMP7 for 20 days (Narayanan et al., 2013). Recent reports demonstrated stepwise induction of UB and/or MM through systematic induction of primitive streak alone, followed by intermediate mesodermal specification (Xia et al., 2013, 2014; Lam et al., 2014; Taguchi et al., 2014; Takasato et al., 2014). These studies performed thorough characterization of cell types obtained at every stage, focusing on obtaining $PAX2^+$ $GATA3^+$ $LHX1^+$ UB cells (Xia et al., 2013) and $SIX2^+$ $PAX2^+$ $OSR1^{+-}GDNF^+$ $HOX11^+$ $WT1^+$ MM cells (Taguchi et al., 2014). Takasato et al. were able to generate UB and MM between 14 and 18 days, whereas Lam et al generated $SIX2^+$ $SALL1^+$ $WT1^+$ cap mesenchyme by 8 days and showed the potency of $PAX2^+$ $LHX1^+$ imtermediate mesodermal cells to generate tubule structures that express Lotus lectin and Ksp-Cadherin after 9 days of differentiation (Lam et al., 2014; Takasato et al., 2014).

Together, these important studies provided proof of principle that most if not all components of the kidney can be induced from PCS. The PSC-derived UB cells can be utilized to generate a ureteric tree or PSC-derived MM cells can be coaxed to produce S-shaped bodies that undergo proximal distal patterning initiating tubulogenesis, giving rise to fetal nephrons in culture. It would also be interesting to use cells derived from the Xia protocol to generate UB and Taguchi protocol to obtain MM and investigate if they interact in a similar manner as these cell types do *in vivo*. Since most kidney diseases involve the damage and loss of podocytes or hypertrophy of tubular epithelial cells, these cell types have a high priority of being derived. On the other hand, the big picture of nephron reconstruction requires other specialized cells including mesangial cells, glomerular endothelial cells, epithelial cells of the loop of Henle, principal cells and intercalated cells that have not yet been procured from PSCs. Induction of terminal differentiation, recapitulation of the architectural context and building of functional nephrons of the kidney are key challenges to be mastered.

Morphogenesis and Patterning

The development of an organism involves not only differentiation of cells, but also their morphogenesis and appropriate patterning to form the architectural context of tissues and organs (Gilbert, 2000). Essentially, cells need to communicate with each other and their microenvironment, by means of growth factors, morphogens, cell adhesion molecules, and mechanoreceptors. The elegant experiments of Holtfreter and Townes studying Xenopus embryo re-aggregation patterns and organization indicated that a whole system of attraction and repulsion phenomena operates between various cell types during development and that information on this system will yield valuable information concerning the segregation of tissues during organogenesis. Holtfreter used the term "tissue affinity" (Gewebeaffinität) to describe this force (Holtfreter, 1939, 1944; Townes and Holtfreter, 1955). Steinberg's differential adhesion hypothesis supported the ideas of Holtfreter with thermodynamic principles (Steinberg, 1970) providing a partial explanation of the self-organizing properties of tissues. Embryonic tissue re-aggregation and adult organ-derived cells have demonstrated this property, e.g., in liver, lung, eye, brain, and thyroid gland (Weiss and Taylor, 1960; Grover, 1961a,b; Ishii, 1966; Hilfer et al., 1968; Stefanelli et al., 1977). The observation of such tissue affinity and self-assembly among PSC-derived progeny flags the success of recapitulating natural embryological behavior *in vitro*.

Toward this extent, one of the first reports of such a phenomenon in mouse PSC-differentiated cells came from the Sasai lab. The appearance of multi-layered cortical neuro-epithelium in serum-free embryoid body-like aggregates in suspension culture, showed a similar pattern as observed during development. This remarkable finding could also be reproduced in hESCs (Eiraku et al., 2008). Further examples of self-organizing tissue morphogenesis were the generation of optic cups from mouse ESCs (Eiraku et al., 2011) and of neural

retina from human ESCs (Nakano et al., 2012). Lancaster et al., developed 3-dimensional self-organizing cerebral organoids from human ESCs, which develop distinct interconnected brain regions (Lancaster et al., 2013). Spence et al. developed self-organizing intestinal organoids from human ESCs, which recapitulated cell types and the stem cell compartment of the intestine (Spence et al., 2011).

Partial self-organization of mouse embryonic kidney cells upon their re-aggregation after dissociation was first achieved by Unbekandt and Davies (2010). The novelty of this system was the introduction of a ROCK inhibitor, which prevented the dissociation-induced apoptosis within single cell suspensions and facilitated a significant recovery of re-aggregated tissues. The Unbekant re-aggregation method has since then been used as a test system to check the capacity of cells (e.g., PSC-derived cells) to integrate into forming tubules or glomeruli of the mouse nephron. While this method proves the property of test cells to contribute to kidney formation, it cannot provide proof of self-organization of PSC-derived renal progenitors. To this end, cases of kidney organogenesis from PSC have been reported by Takasato et al., where 18 days of differentiation of PSC seeded initially on matrigel, develop an $ECAD^+$ ureteric epithelium surrounded by clumps of $SIX2^+$ $WT1^+$ $PAX2^+$ MM cells or $JAG1^+$ $CDH6^+$ renal vesicles (Takasato et al., 2014). Lam et al. also observed appearance of tubule-like structures positive for Lotus lectin (a proximal tubule marker) from SIX2+ cap mesenchyme cell cultures, obtained on day 7 of their differentiation procedure, upon treatment with CHIR99021 (Lam et al., 2014). This was reminiscent of induced metanephric mesenchyme that responds to Wnt signals to undergo mesenchymal-epithelial transition and form renal vesicles *in vivo* (Park et al., 2007; Schmidt-Ott et al., 2007). Meanwhile, the group of Nishinakamura had also obtained evidence of a slightly different nature. They used PSC in the form of EBs for a differentiation protocol that took 8.5 days in mouse ESCs and 14 days in human iPSCs, resulting in $SIX2^+$ $WT1^+$ $SALL1^+$ $PAX2^+$ MM cells that could give rise to tubules and podocytes when induced by mouse embryonic spinal cord (Taguchi et al., 2014). These studies are evidence that a systematic mirroring of embryonic kidney development in PSC derivatives can lead to the formation of organo-typical structures, as summarized in **Table 1**. This brings us a step closer to develop nephrons *in vitro,* as these structures can be coaxed under concentration gradients of growth factors to boost tubulogeneis. Yet, there is clearly a need to enhance protocols to achieve full maturation of nephrons that have on one hand a filtering unit and on the other hand an optimal spatial orientation of tubules and trigger their functionality in terms of electrolyte transport.

A niche for organogenesis was created in post-blastocyst mutant mouse embryos that are genetically precluded from developing a particular organ by Kobayashi et al. The group generated a chimeric animal where injected PSC-derived cells colonized this developmental niche and compensated for the developmental defect to form a donor-induced organ *in vivo*. They injected rat wild-type PSCs into $Pdx1^{-/-}$ mouse blastocysts, generating normally functioning rat pancreas in $Pdx1^{-/-}$ mice. These data constituted proof of principle for interspecific blastocyst complementation and for generation of organs derived from donor PSCs using a xenogenic environment *in vivo* (Kobayashi et al., 2010). On similar lines, the same group injected mouse PSCs in *Sall1*-deficient mice, since *Sall1* is expressed in the metanephric mesenchyme–derived structures in the developing kidney. The kidneys of $Sall1^{-/-}$ chimeric mice were almost entirely composed of EGFP-marked mouse iPSC-derived cells. These iPSC-derived kidneys of $Sall1^{-/-}$ chimeric mice were grossly normal in shape and size. Bladders of these mice were filled with urine, indicating that the iPS-derived kidneys were functional and appropriately connected to the lower urinary tract (Usui et al., 2012). It can be anticipated that human organs can similarly be developed in animal hosts.

REVISITING RENAL DEVELOPMENT—MATURATION OF NEPHRONS

Kidney organoids obtained *in vitro* are comparable to E12.5–E13.5 metanephric mouse kidneys, where in, the ureteric tree

TABLE 1 | Status of lab-grown kidney organoids.

Cell source	Description	References
IN VITRO		
Embryonic cells	Suspension culture of dissociated-reaggregated chick mesonephric cells	Moscona and Moscona, 1952
	Culture at air–medium interface of dissociated- reaggregated mouse metanephric cells	Unbekandt and Davies, 2010
Adult cells	Collagen matrix embedded murine and human renal cells	Joraku et al., 2009; Guimaraes-Souza et al., 2012
PSC- derived cells	2-dimensional culture of 18 day differentiated hPSC	Takasato et al., 2014
	14-day differentiated EBs induced with mouse embryonic spinal cord	Taguchi et al., 2014
IN VIVO		
Embyonic cells	Mouse metanephric cells cultured on CAM of avian embryos	Preminger et al., 1980
	Rat metanephroic cells transplanted in the omentum of a rat	Hammerman, 2002
	Mouse metanephric cells cultivated in mouse lymph node	Francipane and Lagasse, 2015
PSC-derived cells	Mouse embryonic spinal cord-induced EBs transplanted under the kidney capsule of mice	Taguchi et al., 2014
	Sall1- deficient mouse blastocyst, complemented with wildtype mouse PSCs	Usui et al., 2012

has branched and the cap mesenchyme undergoes mesenchymal-to-epithelial transition into renal vesicles, which elongate and undergo patterning to form comma-shaped and S-shaped bodies that are scattered in the cortex. In parallel, the renal stroma, derived from Foxd1$^+$ cells and Flk$^+$ cell derived vasculature, are also contributing to development of kidney architecture (Hatini et al., 1996; Robert et al., 1996; Abrahamson et al., 1998). Formation of renal vasculature is a combination of angiogenesis and vasculogenesis. The lateral branch of the aorta invades the kidney at E12.5 and becomes the renal artery that has 3–4 branches by E13.5. Around E17.5, the arterial tree extends until the cortex due to strong VEGFA signals from developing podocytes in the glomerular zone, leading to the formation of afferent arterioles. Although it has been observed that at around E13-14, endothelial cells migrate into the cleft of glomeruli to form a capillary network, the source of these cells remains elusive (Herzlinger and Hurtado, 2014). Lineage tracing of Tie1/LacZ E11 metanephroi transplanted into a nephrogenic cortex has shown that endothelial precursors exist before the onset of nephrogenesis since the donor tissue showed transgene-expression in glomerular capillary loops (Loughna et al., 1997). A cKIT$^+$ cell population originating from the aorta-gonad-mesonephros hemangioblasts has also been observed during E10.5–E11.5 that are distinct from Foxd1+ stromal cells (Schmidt-Ott et al., 2006), the fate of these cells has not been examined. Foxd1+ stromal derivatives differentiate into peritubular capillary endothelial cells that can be observed around E18.5 (Sims-Lucas et al., 2013) and also secrete factors required for normal nephron and vascular differentiation (Das et al., 2013; Hum et al., 2014).

Rymer et al. performed *in utero* embryonic intra-cardiac injection of tomato lectin (TL) to label perfused blood vessels at different stages of development (E11.5–E17). They found that perfused blood vessels were closely linked to mature nephron structures, while the nephrogenic zone did not show evidence of blood flow. This suggests that terminal differentiation is linked to an oxygen-rich environment, while multipotent nephron progenitors exist under low oxygen conditions (Rymer et al., 2014). This finding is potentially important with respect to tissue engineering, since strategies to establish blood flow and oxygenation within organoids may provide a critical step toward generating mature renal tissues.

BRINGING BLOOD INTO AN ORGANOID—VASCULARIZATION STRATEGIES

PSC-derived tissue specific cell types irrespective of being generated in a 2- or 3-dimensional culture are functionally immature despite having morphological properties of the desired cell type. Essential functions of the kidney, including glomerular filtration, tubular reabsorption, or hormone secretion, essentially dependent on the presence of a vasculature. In addition, the diffusion limit of oxygen in organ cultures is around 150 μm, while the organoids that are currently being developed range from 1 to 2 mm, raising an issue of oxygen deprivation in the core regions gradually leading to tissue decay.

Learning from embryonic vascular development, each organ has progenitors distributed in the primordia that develop into the vasculature of the respective organ. Growth factors acting on these progenitors, including VEGF (Tufro et al., 1999), stem cell factor (SCF) (Schmidt-Ott et al., 2006), and Ang1 (Loughna et al., 1997; Woolf et al., 2009) can be supplemented into culture media to enrich the differentiated progeny. Xinaris et al. used 5-day-organoids derived from E11.5 mouse kidney, dissociated into single cells, and used a protocol involving VEGF treatment and transplantation of these cells under the kidney capsule of athymic rats. This facilitated vascularization with blood vessels from donor (mouse) origin, *in vivo* viability for at least 3 weeks, and glomerular maturation characterized by formation of capillary walls with fenestrated endothelium, podocytes with foot processes and slit diaphragms. Moreover, FITC-conjugated albumin injections were recovered in proximal tubules suggesting successful glomerular filtration and tubular reabsorption in these allografts (Xinaris et al., 2012).

Another approach that could enhance vascularization of organoids that are considerably thick is *in vitro* addition of vascular and perivascular cells. Formation of micro-capillary like structures by endothelial cells prior to transplantation accelerates anastomosis with host vasculature. The first report of *in vitro* pre-vascularized tissues was the generation of a skin equivalent by culturing dermal fibroblasts, keratinocytes and human umblical veil endothelial cells (HUVECs) (Black et al., 1998; Tonello et al., 2003). Stevens et al constructed "tri-cell" cardiac patches containing hESC-derived cardiomyocytes, HUVECs, and mouse embryonic fibroblasts (MEFs) in 1:1:0.5 ratios, respectively (cardio-HUVEC-MEF patches). In sharp contrast to cardio-HUVEC patches, addition of MEFs to human cardiomyocytes and HUVECs resulted in the formation of human CD31-positive endothelial cell networks that morphologically resembled a vascular plexus (Stevens et al., 2009). Similar success has been reported in generating a human liver bud where PSC-derived hepatic progenitors, human mesenchymal stromal cells(hMSC) and HUVECs were co-cultured *in vitro* prior to cranial transplantation in immune-deficient mice (Takebe et al., 2013). As a development to the previous study, Takebe et al. also developed kidney buds by substituting PSC-derived hepatic progenitors with E13.5 kidney derived dissociated cells that could form glomerular like micro-tissues after 8 days of transplantation followed by quick vascularization (Takebe et al., 2015).

A step closer toward a vascularized organoid is the use of scaffolds that are pre-vascularized with HUVECs with or without perivascular cells, followed by seeding of dissociated cells of the organoid or just PSC-derived sorted progenitors. Acellular porcine or rodent kidney matrices have been repopulated with HUVECs lining vessel walls and fetal kidney cells in tubular and glomerular compartments; orthotopic transplantation of the reseeded kidney in single nephrectomized rats showing blood flow through the vasculature and urine production (Song et al., 2013). Miller et al. printed rigid 3D filament networks of carbohydrate glass in the engineered tissue to generate cylindrical networks that can be lined by endothelial cells and perfused,

offers an independent extravascular tissue. Initially, a mixture of glucose, sucrose, and dextran was developed such that it formed a glass when cooled and optically transparent such that it could be used in conjunction with photo-cross-linkable materials. The carbohydrate glass was then extruded through a heated syringe into an interconnected microfluidic vascular network allowed to cool, and a scaffold was cast around it in a mixture of mouse fibroblast cells and photo-cross-linkable ECM pre-polymer. When immersed in water, the carbohydrate quickly dissolved, leaving a hollow network in its place (Miller et al., 2012). Bio-printing and micro-fabrication of cells embedded in hydrogel in a pattern generated from knowledge of renal architecture is an emerging technology that is still in its infancy. Currently, acellular adult kidneys and bio-printing are methods that may provide architectural features similar to that of an adult organ, and hold a promising future for creating vascularized transplantable organs *in vitro*. Current limitations of bioprinting are dimensional, with minimal sizes approaching 30–50 μm, and the availability of adequate materials. Extracellular matrix printing seems to provide some benefit over other polymers in terms of cell survival and differentiation. Its combination with shape controllable polymers, for example to print preformed scaffolds that are then filled with matrix containing cells or cell clusters will perhaps lead to readily generated 3-dimensional and properly tubulized tissues. What remains to be figured out is which cell type can be chosen to repopulate such a pre-vascularized-scaffold. Among various other candidates, would several PSC-derived organoids occupy the matrix and develop a nephron-vascular system or is it sufficient to use a cocktail of PSC-derived terminal cell types and allow them to anchor to the scaffold and attain function?

The simplest method to vascularize the kidney organoid would involve connection to an extrinsic vascular supply. This was first demonstrated by Sariola et al., when they grafted mouse embryonic kidney explants on to chorio-allantoic-membrane (CAM) of a quail and maintained them for 5–10 days. The grafted kidney gave rise to well- developed convoluted secretory tubules and highly branched collecting ducts. Definitive glomeruli were also identified in these grafts by the presence of efferent tubules, visceral and parietal epithelium, capillary tuft, and Bowman's space. These CAM grafts appeared histologically comparable to the 14- 15 -day *in vivo* embryonic metanephros (Preminger et al., 1980). Following this example, Taguchi et al placed mouse-PSC-derived MM cell mass induced by mouse spinal cord beneath the kidney capsule of immunodeficient mice. When harvested after a week, tubulogenesis and glomerular formation was seen as expected and around 84.6% of the glomeruli showed integration of vessels from the host. This demonstrates maturation of the induced EB in presence of a vascular supply (Taguchi et al., 2014).

Ectopic organogenesis has been extended to several sites *in vivo,* since the kidney capsule restricts space availability for the maturating renal primordia. Transplantation of E15 rat metanephroi into omentum of non-immunosuppressed adult rats resulted in a normal kidney structure and function following uretero-uterostomy, surviving as long as 32 weeks (Hammerman, 2002). A recent study by the Lagasse group has reported the successful organogenesis of the kidney using the mouse lymph node as a developmental niche. Some of their findings include the survival of neo-kidneys until the 12th week of transplantation, engraftment and maturation of metanephroi although fluid waste accumulation resulted in graft degeneration. Moreover, nephrectomy of the mice resulted in accelerated organogenesis suggesting a compensatory response (Francipane and Lagasse, 2015). To determine whether the fetal kidney could provide a suitable niche for MSCs to generate renal tissue, Yokote and Yokoo (2013) injected GDNF-expressing human MSCs into rodent whole-embryo cultures at the point of budding. MSC were shown to integrate into the developing fetal kidney forming a chimeric organ leading to a possibility that depletion of animal cells in this chimeric kidney after transplantation into the omentum of human patients would allow further development of the kidney only from human cells (Yokoo and Kawamura, 2009; Yokote and Yokoo, 2013). The result that we aim for in any case, is a kidney that when perfused with blood/ equivalent of body fluids can filter larger proteins and reabsorb electrolytes to secrete a urine concentrate into a functional ureter. Further molecular and cellular investigations of the afore-mentioned experiments of *in vivo* vascularization of kidney organoids (listed in **Table 2**)

TABLE 2 | Strategies to develop a nephro-vascular unit.

Strategy	Description	References
Growth factor induction	VEGF, SCF, Ang-1	Loughna et al., 1997; Tufro et al., 1999; Schmidt-Ott et al., 2006; Woolf et al., 2009
Co-culture with peri-vascular cell types	Fibroblasts and endothelial cells	Black et al., 1998; Tonello et al., 2003
	Mesenchymal stromal cells and endothelial cells	Takebe et al., 2013, 2015
Providing architectural and mechanical support	Endothelialization followed by repopulation with kidney cells of • an acellular organ matrix • 3D printed polymer-based network Suitable for perfusion through endothelial channels	Nakayama et al., 2010; Miller et al., 2012; Song et al., 2013
IN VIVO		
Providing a niche for ectopic organogenesis	CAM	Preminger et al., 1980
	Beneath the kidney capsule of a rodent	Taguchi et al., 2014
	Omentum of a rodent	Hammerman, 2002
	Rodent lymph node	Francipane and Lagasse, 2015

can provide valuable lessons on how the vascular networks are established and can be transferred to *in vitro* experiments conducted on scaffolds, increasing our chances to develop a nephron-vascular unit.

OUTLOOK

The options for generating vascularized tissues suitable for *in vitro* assays or for regenerative medicine have increased greatly in the last decade. Vascularization is achievable *in vitro* by means of preformed natural or artificial printed or casted scaffolds or pre-established endothelialized networks, which can then be populated with tissue cells, depending on the application. The populating cells may lead to further remodeling and capillary formation, which could be directed using switchable expression of angiogenic factors. Alternatively, supplying tissues, cells or organoids undergoing morphogenesis with vascular or lymphatic precursor cells may be useful to enable the tissue to autonomously generate vascularity or to allow faster vascularization post transplantation. The perfusion of the vascular system *in vitro* will become feasible with advanced microfluidics devices and bioreactors for larger organs.

In addition, vascularization of preformed avascular organoids is achievable *in vivo*, by transplantation either ectopically or at the organ specific target site. The aggregation of pre-formed smaller functional tissue units or organoids may be a technique to build larger organs. These smaller units could be either avascular or preconditioned with endothelial cells or suitable scaffolds to quickly vascularize the larger aggregates. The provision of larger vessel adapters to allow direct connectivity to the host's vascular net will provide blood supply early after transplantation. Finally, the generation of embryonic or adult human organs *in vivo* would allow complete vascularization after transplantation to environmental niches that permit continuing organogenesis. These niches could be provided *in vitro* and *in vivo*. Based on this approach, growth of pre-vascular fetal human kidneys in transgenic kidney deficient mice may be used to generate kidney primordia for transplantation and vascularization in adult human hosts. The establishment of a fully humanized kidney *in vivo*, including human derived vasculature and innervation may also be achieved using hMSC in an animal model as demonstrated by Yokote et al. (2015). Similarly, adult kidneys could be generated in donor animals that promote development of fully humanized organs and transplanted or used in perfusion systems.

Striking advances have also been achieved regarding the toolbox for generating human cells that are useful to generate tissue specific organoids. This includes the establishment of well characterized adult and fetal tissue derived primary differentiated and precursor cells, as well as of PSC-derived endothelial, lymphangiogenic, and organ specific cells. Endothelial cells and vasculatures have tissue-specific characteristics, for example in the kidney glomerulus they are highly fenestrated and differentiate in close association with podocytes. Whether iPSC-derived endothelial cells are able to acquire the tissue specific phenotype needs to be investigated. The design of cells with tailored function is another option where the basic technologies are already known. It can be expected that design of cells will advance to the tissue and organoid level including cells with vascularizing functions.

Applications of organoids, which model function and structure of the kidney, are not restricted to understanding human kidney development. They are applicable to study the pathogenesis of genetic diseases as patient –derived PSC can be used for organoid generation. These are equally applicable for drug testing as PSC can be stratified according to patient cohorts and organoids functionally characterized *in vitro* as well as *in vivo* post grafting. Finally, organoids may be applicable in regenerative medicine to replace or repair functional tissue units.

ACKNOWLEDGMENTS

This work was funded by the German Research Foundation [Emmy-Noether grant (to KS), Research Unit 667 grant (to KS), and Research Unit 1368 grant (to KS)] and the Urological Research Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 20 October 2015
doi: 10.3389/fcell.2015.00065

Exosomes as renal inductive signals in health and disease, and their application as diagnostic markers and therapeutic agents

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Cells secrete around 30–1000 nm membrane-enclosed vesicles, of which members of the subgroup between 30 and 100 nm are termed exosomes (EXs). EXs are released into the extracellular space and are widely present in body fluids and incorporated mRNA, miRNA, proteins, and signaling molecules. Increasing amounts of evidence suggest that EXs play an important role not only in cell-to-cell communication but also in various physiological and disease processes. EXs secreted by kidney cells control nephron function and are involved in kidney diseases and cancers. This makes them potential targets for diagnostic and therapeutic applications such as non-invasive biomarkers and cell-free vaccines and for use as drug delivery vehicles. This review provides an overview on the known roles of EXs in kidney development and diseases, including renal cancer. Additionally, it covers recent findings on their significance as diagnostic markers and on therapeutic applications to renal diseases and cancers. The intention is to promote an awareness of how many questions still remain open but are certainly worth investigating.

Keywords: extracellular vesicles, exosomes, kidney development, diagnostic markers, therapeutics, renal disease, renal cancer

OPEN ACCESS

Edited by:
Tomer Avidor-Reiss, University of Toledo, USA

Reviewed by:
Bas W. M. Van Balkom, University Medical Center Utrecht, Netherlands
Gloria Alvarez-Llamas, Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz, Spain

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 July 2015*
Accepted: *05 October 2015*
Published: *20 October 2015*

Citation:
Krause M, Samoylenko A and Vainio SJ (2015) Exosomes as renal inductive signals in health and disease, and their application as diagnostic markers and therapeutic agents. Front. Cell Dev. Biol. 3:65. doi: 10.3389/fcell.2015.00065

INTRODUCTION

Cells from various organisms, including all the eukaryotes and many prokaryotes, release extracellular vesicles of different types into their environment. The term exosomes (EXs) describes those vesicles which are of endosomal origin. They are small (30–100 nm) membrane-enclosed vesicles which are secreted by various cell types, and can be found in biological fluids such as blood, semen, saliva, and urine (reviewed by Raposo and Stoorvogel, 2013; Yanez-Mo et al., 2015). Initially these were considered to take part in the cell's waste management but evidence has accumulated that they are instrumental in intercellular and system (humoral)-level communication in organisms. The striking property of EXs is that they can transfer important compounds such as membrane and cytosolic proteins, lipids, mRNA, miRNA (Valadi et al., 2007), and even DNA between cells. Thus, they provide a novel but apparently evolutionary ancient platform for cell-to-cell and tissue interactions (Valadi et al., 2007).

Even though the putative developmental role of EXs remains in most respects unclear, it is emerging that at least many of the key developmental signaling pathways are coordinated by EXs (Raposo and Stoorvogel, 2013; Urbanelli et al., 2013). Kidney cells, for instance, appear to release vesicles presumably in a developmentally regulated manner, and the currently available data suggest

that EXs may initiate and regulate organogenesis. In addition, they may also take part in processes such as regeneration and the development of diseases such as cancer. Interestingly, kidney-derived vesicles may also have an impact on the cells of certain other organs (Grange et al., 2011) and even the immune system. The aim of this review is to summarize the roles that have been demonstrated for EXs in kidney development and disease, especially in kidney tumorigenesis.

EXOSOMES AS RENAL INDUCTIVE SIGNALS, DIAGNOSTIC MARKERS, DISEASE, AND THERAPEUTIC AGENTS

Biogenesis and Signal Transduction

The biogenesis of EXs is a complex intracellular process that forms part of the endosomal cell sorting machinery, in which an array of regulatory factors has been identified (Stoorvogel et al., 2002; Théry et al., 2002; Kowal et al., 2014). Upon the initiation of EX assembly an early endosome is formed by an inward budding of the cellular membranes via endocytosis. In association with this budding the endosomal membrane undergoes a second inward folding to generate further smaller vesicles inside the endosomal lumen. The process is collectively called the formation of multivesicular bodies (MVB; **Figure 1**).

The machinery known as the Endosomal Sorting Complex Required for Transport (ESCRT) is involved in the formation of MVBs, although it also entails an ESCRT-independent mechanism involving proteins such as tetraspanins and lipids (for details, see recent reviews by Hanson and Cashikar, 2012; Henne et al., 2013; Colombo et al., 2014; Kowal et al., 2014). The assembled MVBs can then fuse either with lysosomes, leading to their degradation, or alternatively with the cellular plasma membrane (PM), which releases their vesicles, called from here on exosomes (EXs), into the extracellular milieu of the cell. The fusion of MVBs with the PM was first described in the hematopoietic system, namely in differentiating red blood cells (Pan and Johnstone, 1983), and thereafter in several other cell types such as B-cells, T-cells, dendritic cells (Escola et al., 1998; Zitvogel et al., 1998; Blanchard et al., 2002), mast cells (Raposo et al., 1997), and yeast cells (Henne et al., 2013). The biogenesis of EXs has also been described in epithelial cells, including those of the kidney (Knepper and Pisitkun, 2007), being the cells that line the renal tubule lumen (Pisitkun et al., 2004).

On their release EXs interact with a recipient cell in several ways. A schematic overview of the biogenesis of EXs and how they enter target cells and interact with them is depicted in **Figure 1**. As one mode of interaction, EXs bind to the target cell via membrane receptors such as the Major histocompatibility complex (MHC) that mediates binding to T-cells (Denzer et al.,

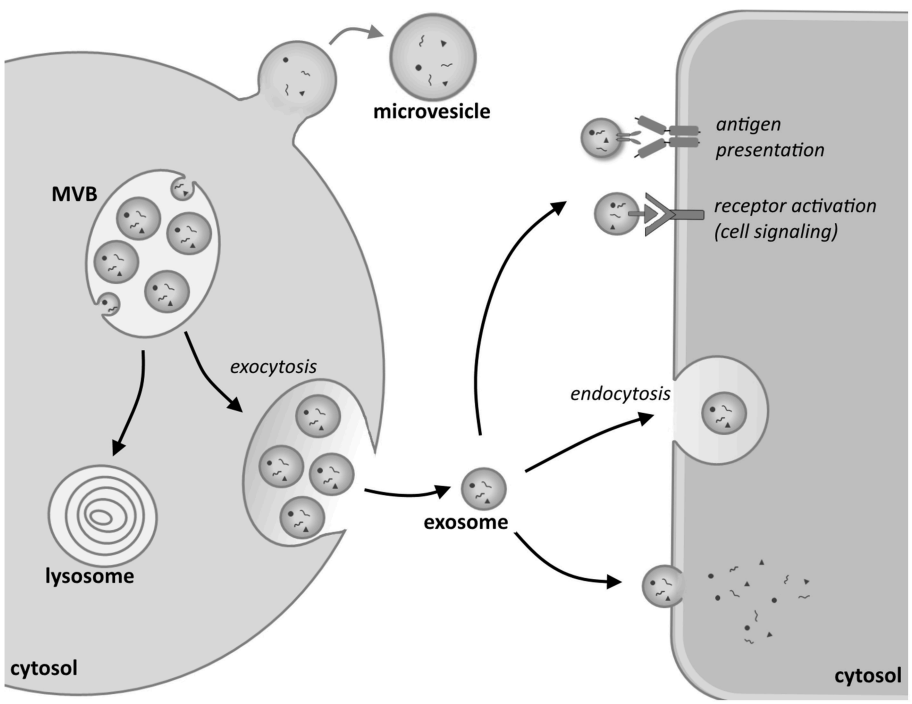

FIGURE 1 | Biogenesis and uptake of exosomes. Inward budding of the membrane of an endosomal vesicle in the cytosol of the donor cell leads to the formation of multivesicular bodies (MVBs). These can either turn into lysosomes, whereupon their content will be degraded, or fuse with the plasma membrane, releasing their content into the extracellular environment. These vesicles are called exosomes. By contrast, larger microvesicles are formed when the cell membrane directly buds outwards, giving rise to micelles. Exosomes can interact with recipient cells in various ways. Signaling can be initiated by an antigen-antibody interaction in the recipient cell or by the activation of a receptor on the cell membrane of the target cell. Exosomes can also be taken up by endocytosis or fuse directly with the plasma membrane. Upon fusion their cargo is released into the cytosol of the target cell.

2000; Nolte-'t Hoen et al., 2009). Another way in which EXs bind is via recognition of the ligands on their lipid bilayer by means of specific cell surface receptors. Indeed, many common ligand/receptor pairs such as recognition by integrins or tetraspanins have been identified. The capacity of an EX to bind to a given cell depends to a great deal, however, on the cellular content, and also the characteristics of the donor cell from which the EXs originated. The most prominent binding mechanisms have been reviewed recently by Colombo et al. (2014) and are therefore, not reviewed here. Besides receptor-mediated binding, the entry of EXs into a target cell may involve endocytosis (Morelli et al., 2004), phagocytosis (Feng et al., 2010; Christianson et al., 2013; Yanez-Mo et al., 2015), or pinocytosis (Parolini et al., 2009; Théry et al., 2009; Yanez-Mo et al., 2015). These processes can also be receptor-mediated, aided by several other proteins such as dynamin (Fitzner et al., 2011) and clathrin (Mulcahy et al., 2014; Tian et al., 2014), which regulate entry. In the case of kidney-derived cells such as COS-7, the cells seem to internalize EX through lipid raft-mediated endocytosis which is negatively regulated by caveolin-1 (Svensson et al., 2013). It is currently poorly understood whether EX uptake mechanisms are common or specific to each cell type (Gildea et al., 2014). Furthermore, there is also some debate as to how widely certain EXs can influence cell behavior (White et al., 2006; Svensson et al., 2013).

Exosomes in Kidney Physiology

The currently available data suggest that the extracellular vesicles are coupled to normal development and various diseases. Given that EXs contain key regulatory signals such as mRNA and miRNA and can transfer them from one cell type to another, they may have a profound influence on target cell homeostasis (Montecalvo et al., 2012; Stoorvogel, 2012; Tomasoni et al., 2012; Zhang et al., 2015b). The capacity of EXs to transport miRNA is of particular importance when considering participation in developmental control in general (Tang et al., 2007). Even though it still remains unknown whether EXs indeed play a crucial role in morphogenesis, the current results suggest that at least urinary EXs, besides being involved in the secretion of senescent proteins as exocytic waste (Pisitkun et al., 2004; Knepper and Pisitkun, 2007) may also have other roles (van Balkom et al., 2011; Fang et al., 2013). In the light of current data, the systemic serum containing EXs cannot cross the kidney filter within the glomerulus under normal conditions (Pisitkun et al., 2004; Gildea et al., 2014), but whether this changes in the presence of kidney anomalies remains open.

Proteomic studies of urinary EXs have identified proteins that characterize certain nephron segments such as the glomerulus and Henle's loop. A summary is given in **Table 1** (Pisitkun et al., 2004; Miranda et al., 2010; Dear et al., 2013). The available data suggest that most, if not all, of the nephron cell types have the capacity to secrete vesicles. EXs may play an important role in mediating cell-to-cell communication along the nephron with potential significance for kidney performance, and it has been shown that the function of the EXs within the nephron may be the adaptation of nephron function to changes in physiology, i.e., in homeostasis. This possibility is based on the observation that different segments of the nephron secrete and take up EXs differentially (Dimov et al., 2009). Renal EXs can also transfer functional molecules such as aquaporin-2 between cells (Street et al., 2011), although their uptake capacity becomes restricted in the fully matured adult kidney. This is probably caused by accumulation of the tubular Tamm-Horsfall protein, which prevents contacts between EXs and the tubular luminal cells unless this protein is degraded locally (van Balkom et al., 2011). Hence, the luminal epithelial cells of the nephron apparently secrete the EXs found in urine. Furthermore, proteins which are specifically associated with the urinary bladder and prostate gland have also been found in urine (Musante et al., 2014), although further studies are needed to provide conclusive evidence of this.

When considering the embryonic kidney and the potential of EXs for taking part in its developmental programming, many of the proteins known to control organogenesis, including growth factors (see the recent review by Krause et al., 2015) are in fact found in the EXs that have been characterized from a variety of cell lines (**Table 2**). The Wnt-family members and their signal transduction pathway are critical for kidney development, and interestingly, several Wnt proteins and their downstream factors such as β-catenin are associated with EXs and can also mediate activation of the pathway (**Table 2**; Zhang et al., 2015a). It can be speculated that a panel of key developmental signals may also be associated with and transported to the target cells via EXs during kidney development.

If this is the case, then EXs constitute a new critical mechanism in the control of kidney development by transferring and integrating key inductive signals. Thus, their presence during kidney development should be explored further: Which cells secrete EXs under which environmental conditions, and do these EXs populations differ one from another? Further analysis should then reveal details of their content, transport mechanisms and physiological roles during kidney development.

The putative role of EXs has recently been explored in Madin-Darby canine kidney cells (MDCK; Kwon et al., 2014). When these cells are subjected to the hepatocyte growth factor (HGF) their proliferation is stimulated and eventually tubular cysts form. Changes in the expression of a specific G protein-coupled receptor, GPRC5B, are associated with EXs being secreted by these cells. This protein is also up-regulated in tubulogenesis, while the exosomal delivery of GPRC5B induces extracellular signal-regulated kinase 1/2 (Erk1/2). As the GPRC5B is expressed in the ureteric bud of the embryonic kidney, this may suggest that it also plays a role in organogenesis.

The Role of Exosomes in Kidney Regeneration and Diseases

While the role of kidney-derived EXs in physiological processes remains poorly investigated, EXs have been found to exercise beneficial or adverse functions in the development of several kidney diseases (Borges et al., 2013a; Fang et al., 2013). For example, vesicles derived from mesenchyme stem cells (MSCs) or endothelial stem cells can promote kidney regeneration (Borges et al., 2013a). The positive impact of MSCs on both acute and chronic kidney injury (AKI and CKI) was first attributed to

TABLE 1 | Proteins found in human urinary exosomes that are specific to or enriched in given regions of the kidney.

Kidney region	Gene symbol	Full gene name	Species	Exosome sample	Identified molecule	Associated diseases
Glomerulus	PODXL	Podocalyxin-like Protein	Homo sapiens	Urine	Protein	Diabetic nephropathy
	NPHS2	Podocin	Homo sapiens	Urine	mRNA	Focal segmental glomerulosclerosis, nephrotic syndrome [MIM: 600995]
	LGALS1	Galectin-1	Homo sapiens	Urine	mRNA	–
	HSPG2	Heparan Sulfate Proteoglycan 2	Homo sapiens	Urine	mRNA	Schwartz-Jampel syndrome type 1 [MIM: 255800]
1st convoluted tubule	gp330 precursor	Glycoprotein 330 Precursor	Homo sapiens	Urine	Protein	Renal aminoglycoside accumulation and nephrotoxicity, Donnai-Barrow syndrome
	CUBN	Cubilin (Intrinsic Factor-Cobalamin Receptor)	Homo sapiens	Urine	Protein, mRNA	Megaloblastic anemia 1 [MIM: 261100]
	AQP1	Aquaporin1	Homo sapiens	Urine	protein, mRNA	Nephrogenic diabetes insipidus, Aquaporin 1 deficiency, Colton-Null [MIM: 110450]
	LRP2	Megalin	Homo sapiens	Urine	mRNA	Heymann nephritis, proteinuria
	CA4	Carbonic Anhydrase 4	Homo sapiens	Urine	mRNA	Diabetic nephropathy, Proximal renal tubular acidosis [MIM: 114760]
	ANPEP	Alanyl Aminopeptidase	Homo sapiens	Urine	protein	Hypertension [MIM: 151530]
	NAPSA	NapsinA	Homo sapiens	Urine	Protein	Kidney carcinoma, renal neoplasms
	CLCN 5	Chloride Channel Protein 5	Homo sapiens	Urine	mRNA	Dent's disease
	GGT1	γ-glutamyltransferase	Homo sapiens (male)	Urine	Protein	–
	APN	Aminopeptidase N	Homo sapiens (male)	Urine	Protein	–
Henle's loop	AQP1	Aquaporin 1	Homo sapiens	Urine	Protein	Nephrogenic diabetes insipidus, Aquaporin 1 deficiency, Colton-Null [MIM: 110450]
	UMOD	Uromodulin	Homo sapiens	Urine	Protein	Hyperuricemic nephropathy, Medullary cystic kidney disease-2 (MCKD2) [MIM: 603860], familial juvenile hyperuricemic nephropathy (FJHN) [MIM: 16200]
	THP	Tamm-Horsfall Protein	Homo sapiens (male)	Urine	Protein	Mckd2 [mim: 603860], fjhn [mim: 16200]
	CD9	Cluster of Differentiation (Tetraspanin)	Homo sapiens (male)	Urine	Protein	–
	BDKRB1	Bradykinin B1 Receptor	Homo sapiens	Urine	mRNA	–
	CALCR	Calcitonin Receptor	Homo sapiens	Urine	mRNA	Kidney stone disease
	SCNN1D	Amiloride-sensitive Sodium Channel Subunit Delta	Homo sapiens	Urine	mRNA	–
2nd convoluted tubule	SLC12A3 (NCC)	Thiazide-sensitive Na-Cl Cotransporter	Homo sapiens	Urine	Protein	Gitelman syndrome [MIM: 263800]
Collecting ducts	AQP2	Aquaporin 2	Homo sapiens	Urine	mRNA	Nephrogenic diabetes insipidus type 1 [MIM: 222000] [MIM: 125800]
	ATP6V1B1	V-ATPase B1 Subunit	Homo sapiens	Urine	mRNA	Distal renal tubular acidosis [MIM: 267300]
	SLC12A1	Kidney-specific Na-K-Cl Symporter	Homo sapiens	Urine	mRNA	Bartter-Syndrome type 1, 2, 3 [MIM: 601678, 241200, 607364]
	MUC1	Mucin-1	Homo sapiens (male)	Urine	Protein	Renal cell carcinoma, Medullary cystic kidney disease type 1 (MCKD1) [MIM:174000]
	RHCG	Rh type C glycoprotein	Homo sapiens (male)	Urine	Protein	–

Data compiled from Pisitkun et al. (2004), Gonzales et al. (2009), Miranda et al. (2010), Dear et al. (2013), and Musante et al. (2014) and the Online Mendelian. Inheritance in Man OMIM® website (http://www.omim.org/, see MIM numbers for reference).

TABLE 2 | Genes of importance during kidney development found in exosomes of various origins.

Gene symbol	Full gene name	Species	Exosome sample	Identified molecule	References	ExoCarta ID (Mathivanan and Simpson, 2009)
Wnt4	Wingless-type MMTV Integration Site Family, Member 4	Homo sapiens	Umbelical cord mesenchymal stem cells	protein	Zhang et al., 2015a	No
Wnt11	Wingless-type MMTV Integration Site Family, Member 11	Mus musculus	Mast cells	mRNA	Valadi et al., 2007	ExoCarta_22411
Notch2	Neurogenic locus notch homolog protein 2	Homo sapiens	Ovarian cancer cells	Protein	Liang et al., 2013	ExoCarta_4853
		Homo sapiens	Colorectal cancer cells	Protein	Demory Beckler et al., 2013	ExoCarta_4853
		Bos taurus	Milk	Protein	Reinhardt et al., 2013	ExoCarta_513730
BMP4	Bone Morphogenetic Protein 4	Homo sapiens	Colorectal cancer cells	Protein	Demory Beckler et al., 2013	ExoCarta_652
FGFR1	Fibroblast Growth Factor receptor 1	Homo sapiens	Ovarian cancer cells	Protein	Liang et al., 2013	ExoCarta_2260
OSR1	Oxidative Stress responsive 1	Homo sapiens	Ovarian cancer cells	Protein	Liang et al., 2013	ExoCarta_9943
		Homo sapiens	Thymus	Protein	Skogberg et al., 2013	No
		Homo sapiens	Urine	Protein	Gonzales et al., 2009	No
		Rattus norwegicus	Reticulocytes	Protein	Carayon et al., 2011	ExoCarta_316064
WT1	Wilms Tumor 1 Homolog	Mus musculus	Mast cells	mRNA	Valadi et al., 2007	ExoCarta_22431
					Ranghino et al., 2014	No
β-catenin	Cadherin-associated protein beta	Mus musculus	Dendritic cells	Protein	Chairoungdua et al., 2010	No

their role in directly replacing renal tubular cells, but later it became clear that these cells rather provide paracrine support for endogenous regeneration (Biancone et al., 2012). At present the role of MSCs has been assigned in part to the secretion of EXs (Camussi et al., 2010; Biancone et al., 2012; Borges et al., 2013a), e.g., in that MSC-derived EXs can enhance regeneration of the rat kidney epithelium when injured by ischemia-reperfusion (Gatti et al., 2011). This EX-mediated recovery involves cell-to-cell transfer of mRNAs and/or microRNAs and may be connected with the inhibition of renal cell apoptosis and the stimulation of tubular epithelial cell proliferation. Moreover, by reducing the acute injury, the EXs also protected the rats from later CKI development 6 months after the operation (Gatti et al., 2011).

Other studies have reported a regenerative potential in microvesicles (MVs) produced by bone marrow MSCs in glycerol-induced (Bruno et al., 2009), cisplatin-induced (Bruno et al., 2012), and gentamicin-induced (Reis et al., 2012) AKI models *via* a mechanism dependent on RNA delivery. Here the protective effect of EXs was mainly ascribed to an increase in surviving tubular cell proliferation (Bruno et al., 2009; Reis et al., 2012) and a decrease in tubular epithelial cell apoptosis (Bruno et al., 2009, 2012; Reis et al., 2012). These effects are thought to occur via the up-regulation of anti-apoptotic genes and down-regulation of genes involved in the execution phase of cell apoptosis (Bruno et al., 2012).

It has also been found that the development of chronic tubular injury is inhibited by multiple injections of MVs, while the effect of a single injection was not sufficient to prevent CKI (Bruno et al., 2012). Like bone marrow MSC-derived EXs, human umbilical cord MSC-derived EXs also demonstrated a protective effect on cisplatin-induced nephrotoxicity *in vivo* and *in vitro*, whereas human lung fibroblast-derived EXs did not (Dorronsoro and Robbins, 2013; Zhou et al., 2013a). Zhou et al. (2013a) demonstrated that these EXs can reduce cisplatin-mediated renal oxidative stress and apoptosis in rats *in vivo* and increase the proliferation of renal tubular epithelial NRK-52E cells in culture. They also showed that human umbilical cord MSC-derived EXs can reduce Bax (bcl-2-like protein 4) level and increase Bcl-2 (B-cell lymphoma 2) in order to inhibit apoptosis and stimulate Erk1/2, thereby increasing proliferation after cisplatin-induced injury in the kidney. Another group has shown that EXs isolated from peripheral blood-derived endothelial progenitor cells can prevent AKI in an ischemia-reperfusion rat model (Cantaluppi et al., 2012). In this case the miRNAs that modulate proliferation, angiogenesis and apoptosis were found to be responsible for the protective effects of EXs.

Kidney epithelial cells are another source of the EXs involved in kidney regeneration. It was found that administration of the epithelium-derived exosomal ATF3 (activating transcription factor 3) mRNA attenuates ischemia/reperfusion-induced kidney injury by inhibiting monocyte chemotactic protein-1 (MCP-1)-induced macrophage infiltration (Chen et al., 2014b). While all the above-mentioned studies demonstrate beneficial effects of EXs on kidney regeneration, it was found on the other hand that EXs produced by injured proximal tubular epithelial cells in a murine model of hypoxic kidney fibrosis after unilateral ureteral obstruction can initiate tissue repair/regenerative responses and activate fibroblasts, leading to fibrosis (Borges et al., 2013b). Fibroblast proliferation and the production of matrix proteins were particularly dependent on EXs delivering TGF-β1 (transforming growth factor β1) mRNA (Borges et al., 2013b).

Interestingly, certain *in vitro* studies published more than 20 years ago demonstrated that renal brush border-derived MVs about 100 nm in diameter can induce and promote calcium

oxalate crystallization (Nagasawa et al., 1992; Anderson et al., 2010), which is one of the features of nephrolithiasis (kidney stone formation), a pathological kidney condition leading to fibrosis and chronic renal failure (Anderson et al., 2010).

Since, the endothelial cells are connected to the control of blood flow, pressure and clotting, they are prime targets when considering the development of EX-based therapies, and also the treating of renal diseases (van Balkom et al., 2011). It has been shown that circulating levels of endothelial-derived MVs are significantly higher in chronic renal failure patients than in healthy subjects (Faure et al., 2006). Moreover, blood levels of these MVs correlate with endothelial dysfunction and arterial stiffness in end-stage renal failure hemodialysis patients (Amabile et al., 2005). In contrast to endothelium-derived MVs, those produced by platelets or erythrocytes do not seem to be connected with endothelial dysfunction (Amabile et al., 2005). In another study it was found that the number of endothelial-derived microparticles was inversely correlated with brachial artery and aortic laminar shear stress values in end-stage renal disease patients with a high cardiovascular risk (Boulanger et al., 2007).

It may be assumed that the action of cell-damaging agents such as low shear stress and increased arterial stiffness contributes to endothelial apoptosis through a substantial release of endothelial MVs (Amabile et al., 2005; Boulanger et al., 2007). The pathogenetic effects of EXs and other circulating microparticles in promoting vascular calcification and sclerosis in chronic kidney disease (CKD) are nevertheless not yet clearly defined (Anderson et al., 2010; Fang et al., 2013). Rather surprisingly, Neal et al. (2011) found that the levels of circulating miRNAs in patients with different stages of chronic kidney failure, including those receiving hemodialysis treatment, were reduced by comparison with patients having mild renal impairment or normal renal function. This observation might be explained by the fact that many circulating miRNAs are bound to Argonaute 2-containing protein complexes (Arroyo et al., 2011) or to high-density lipoproteins (Vickers et al., 2011) rather than within EXs. It has been demonstrated in a rat model of CKD induced by 5/6 nephrectomy that the administration of conditioned medium from embryonic MSC has a therapeutic effect, whereas MSC-derived EXs tested in the same experimental setting showed no protective effect on the kidney (van Koppen et al., 2012).

Exosomes in Kidney Cancer

Although tumor cells secret large amounts of various MVs that enter the blood and other body fluids (Lee et al., 2011; Azmi et al., 2013), the isolation of cancer EXs from patients remains a serious problem due to the lack of specific markers that can distinguish cancer-derived from non-cancer-derived EXs. One such marker that has been identified recently is glypican-1, detected on EXs derived from the serum of patients with pancreatic cancer but not on EXs from healthy subjects or subjects with chronic pancreatitis (Melo et al., 2015). Various biological roles have been proposed for EXs in cancer, such as the expulsion of key proteins and miRNA from cells, the removal of anti-cancer drugs and the release of signaling and regulatory molecules (Lee et al., 2011; Azmi et al., 2013). The properties of EXs may also enable them to take part in the control of cell proliferation, cell survival, angiogenesis, metastasis and immune response (Azmi et al., 2013; Fang et al., 2013). EX-released factors promote stromal remodeling and hypoxia-mediated epithelial mesenchyme transformation, which is critical for the evolution of cancer (Nieto, 2011). EXs can also stimulate the proliferation of fibroblasts by causing a desmoplastic reaction, and they can induce immune escape mechanisms by suppressing antigen-specific immune responses and by up-regulating immunosuppressive cell differentiation and the functioning of these cells (Azmi et al., 2013; Minciacchi et al., 2015). For these reasons EXs form a critical aspect of tumorigenesis.

Much less is known at present about the roles of EXs in kidney tumorigenesis, however. The relevant findings are that the vesicles released by renal carcinoma stem cells (rCSCs) derived from a tumor-bearing patient can trigger angiogenesis and promote lung metastasis when studied with the SCID (immunocompromised severe combined immunodeficient) mouse model (Grange et al., 2011). Grange et al. (2011) showed that rCSCs are secreted by a subset of tumor-initiating cells characteristically expressing the mesenchymal stem cell marker CD105, and that EXs derived from these rCSCs were able to stimulate the growth and invasiveness of normal HUVEC (human umbilical vein endothelial cells). In addition, these EXs increased the formation of capillary-like structures in culture and in induced vessel formation when cells treated with them were grafted into SCID mice.

More detailed molecular characterization of the CD105-positive EXs pointed to significant differences in mRNA and miRNA content as compared with EXs that were negative for CD105. Consistent with their properties, the rCSC-derived EXs have mRNAs that encode for proangiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin-1, ephrin A3, and matrix metalloproteinases (MMP)-2 and -9. Similarly, these were reported to be absent from the CD105-negative tumor cell-derived EXs. In addition to these findings, miRNAs that have been reported to be involved in biological processes such as the control of transcription, cell adhesion and cell proliferation were enriched in CD105-positive EXs (Grange et al., 2011, 2014).

Another indication that EXs derived from kidney cancer cells may be involved in the cancerous state and its severity, including the formation of metastases, came from the work of Tauro et al. (2013), who performed a proteomic analysis by comparing EXs released by normal and oncogenic H-Ras (21D1) transfected MDCK cells. While the control and 21D1-MDCK cell-derived EXs were similar in their morphology, the 21D1-derived EXs had high levels of proteases, annexins, integrins, and other secreted proteins which are typically associated with the formation of premetastatic niches. The ability of primary cancer to make changes in normal tissue located in a pre-metastatic niche prior to the arrival of metastasizing cells is an important feature that facilitates sustained cancer growth (Wels et al., 2008).

The role of EXs in the "education" of normal cells toward a pro-metastatic phenotype has been demonstrated not only for renal cell carcinoma but also for other cancers, including melanomas (Peinado et al., 2012) and breast cancer (Fong et al., 2015). Elsewhere, the transfection of MDCK cells with oncogenic H-Ras has been found to induce the release of EXs that contain factors known to control nuclear assembly, transcription, splicing, and translation. The most abundant protein in the 21D1-derived EXs was the Y-box-binding protein (YBX1), which is a DNA and RNA- binding transcription factor involved in DNA replication, DNA repair, transcription, pre-mRNA splicing, and mRNA translation (Eliseeva et al., 2011).

Several studies have been conducted employing 786-0 human renal adenocarcinoma cells and targeted EXs in carcinogenesis (Zhang et al., 2013; Chen et al., 2014a; Du et al., 2014). Like the rCSC-derived EXs (Grange et al., 2011) the 786-0-derived ones promote tubulogenesis in HUVECs (Human umbilical vein endothelial cells; Zhang et al., 2013), and it has been speculated that the pro-angiogenic effect of these renal EXs may be mediated via down-regulation of hepaCAM (hepatic and glial cell adhesion molecule), a hepatocyte cell adhesion molecule, and up-regulation of VEGF. In turn, it has been shown that the EX-mediated down-regulation of hepaCAM expression is in effect mediated by Akt phosphorylation connected with the enhanced renal carcinoma cell proliferation (Jiang et al., 2014). Collectively, the 786-0-derived EXs enhance cell migration, invasion and chemokine receptor type 4 (CXCR4) and MMP-9 expression and concurrently reduce the adhesion of 786-0 cells (Chen et al., 2014a).

A comprehensive report on the influence of EXs on renal carcinoma 786-0 cells has been published recently by Du et al. (2014), who investigated the putative effects of EXs released by human Wharton's jelly mesenchyme stem cells (hWJ-MSCs). These EXs promoted cell proliferation, cell migration, and progression of the cell cycle from G0/G1 to the S phase and the HGF/c-Met, Akt, and Erk1/2 pathways in these cells. Moreover, the hWJ-MSCs-derived EXs stimulated tumorigenesis in the 786-0-cells and also enhanced tumor size. At the molecular level, the EXs induced cyclin D1, MMP-2, and MMP-9 expression in a BALB/c nude mouse model (Du et al., 2014). Meanwhile, RNase pre-treatment abrogated these exosomal effects, indicating that the RNA delivered via EXs serves as a crucial mediator (Du et al., 2014).

Little is known as to whether the cells in various organs take up the cancer-derived EXs selectively. Rana et al. (2012) found that small differences in the EX-tetraspanin complexes that originated from different rat tumor cell lines greatly influenced the cell type to which the EXs were targeted *in vitro* or *in vivo*. When tetraspanin-8+ EXs were monitored after 24 h of injection they had been taken up by the pancreas and lung cells, whereas certain large vessels and the kidneys showed a lower abundance of such EXs. Moreover, the liver and gut cells were for the most part negative. These data thus suggest some target cell selectivity among EXs that are secreted *in vivo*.

Interestingly, EXs engineered to express a chimeric tetraspanin-8 in which the N-terminal region was swapped for a domain from the CD9 protein were readily taken-up by kidney cells but not by other organs, whereas EXs engineered to contain tetraspanin-8 that was fused to an integrin β4 was targeted preferentially to lung, kidney and gut cells. It is significant that also within an organ, EXs seem to be taken up preferentially by specific cell types depending on the exosomal protein content. One indication of this is the fact that tetraspanin-8; integrin β+ fusion EX products could be identified in only the kidney glomeruli (Rana et al., 2012).

There are several reports stating that EXs produced by kidney tumor cells are coupled to cancer-associated immune suppression (Yang et al., 2013; Diao et al., 2015; Gu et al., 2015). As an example of this, EXs purified from human kidney adenocarcinoma ACHN cells inhibited proliferation and induced apoptosis of Jurkat-immortalized T-cells while reducing *in vitro* interleukin-2 (IL-2), IL-6, IL-10 and interferon-γ production (Yang et al., 2013). In the light of these findings, the authors proposed that the Fas ligand within the tumor-derived EXs must be responsible for exosomally induced T-cell death.

There is considerable evidence that EXs play a role in renal tumor progression *in vivo*. The survival of mice inoculated with renal adenocarcinoma Renca cells, which go on to generate tumors, was reduced to some degree in the presence of Renca-derived EXs in the assay (Gu et al., 2015), and similar data have been reported by Diao et al. (2015), leading them to propose that the heat shock protein HSP70 present in EXs derived from cancer cells promotes the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs), possibly via an increase in Stat3, a signal transducer and activator of transcription 3-phosphorylation.

Besides suppressing the immune system, EXs can also do exactly the opposite. Certain tumor-derived EXs induce an immune response. This suggests that EXs may even offer opportunities for developing individualized tumor immunotherapies (Greening et al., 2015). Given the fact that tumor-derived EXs also contain immunosuppressive molecules that reduce their immunogenicity, it is important to learn in detail how these properties are regulated, opening new avenues for valuable therapeutics. Along these lines, EXs that are secreted by IL-12-expressing RC-2 human renal cancer cells exhibited greater anti-tumor effects than EXs derived from "wild type" renal cells or cytocine-supplemented IL-12 alone (Zhang et al., 2010). Such IL-12-containing EXs efficiently induced cell proliferation, the release of interferon-gamma and the specific cytotoxic effects of T-cells derived from cultured human peripheral blood cells.

It is also of interest that the mice that had tumors derived either from the mouse myeloid leukemia line WEHI3B or renal carcinoma Renca cells survived longer if they had been vaccinated beforehand with the EX-loaded dendritic cells from the tumor (Gu et al., 2015). In other words, the tumor cell-derived EXs seemed to be superior to the tumor lysates as a source of antigen. Interestingly, the immunosuppressive features of EXs do not detract from their capacity to serve as an antigen source for the dendritic cells (Gu et al., 2015). These properties make the EXs very promising components for the development of novel cancer therapies.

Kidney/Urinary Exosomes as Diagnostic Biomarkers and Therapeutic Agents

EXs have a great potential for use as valuable diagnostic biomarkers, especially in the case of monitoring kidney malfunction. Since, many reviews have been published on the analysis of urinary EXs and their potential as diagnostic markers for kidney disease, injury, and transplant rejection, this topic will be covered in the present review only briefly (Knepper and Pisitkun, 2007; Dimov et al., 2009; Dear et al., 2013; Properzi et al., 2013; Musante et al., 2014; Ranghino et al., 2014; Salih et al., 2014).

As discussed, the urinary EX components can be assigned to specific nephron segments, the glomerulus, the proximal/distal tubule, Henle's loop and the collecting duct (**Table 1**; Pisitkun et al., 2004; Miranda et al., 2010; Dear et al., 2013; Musante et al., 2014). Many of these proteins can be associated with certain diseases, but they are not all necessarily linked directly to the kidney. The level of GPRC5B in the urinary EXs correlates with AKI, so that its values are elevated in cases of AKI by comparison with normal healthy subjects (Kwon et al., 2014), making GPRC5B a candidate diagnostic marker for AKI. Furthermore, levels of Fetuin-A were also found to be elevated in patients suffering from AKI (Zhou et al., 2006).

The review by Ranghino et al. (2014) summarizes several suitable urinary exosomal biomarkers for glomerular and tubular damage, including Wilms Tumor 1 Homolog (WT1), ATF3, and Neutrophil Gelatinase-Associated Lipocalin (NGAL). WT1 was found in urinary exosomes of patients who suffer from focal segmental glomerulosclerosis (FSGS; Zhou et al., 2008, 2013b) and in most diabetic patients (Kalani et al., 2013). ATF3 is another marker for AKI alongside Fetuin A and GPRC5B (Zhou et al., 2008). NGAL levels were elevated in patients with delayed graft function after kidney transplantation (Alvarez et al., 2013), and several protein markers have also been found in cases of diabetic nephropathy (Zubiri et al., 2014) and prostate (Lu et al., 2009; Mitchell et al., 2009) and urine bladder cancer (Smalley et al., 2008; Blackwell et al., 2014).

Some markers that classify a person as healthy have also been identified, and these may be of diagnostic value in cases of kidney regeneration. Promonin-1 (CD133) serves as such a marker, for example, as it is lost in the urine at the end stage of renal disease (Dimuccio et al., 2014), and additional markers have been defined for a panel of kidney diseases that include diabetic nephropathy (Musante et al., 2014), cardio-renal syndrome (Gonzalez-Calero et al., 2014), autosomal-dominant polycystic kidney disease (ADPKD; Fang et al., 2013; Ben-Dov et al., 2014) and Gitelman's and Bartter syndromes (Corbetta et al., 2015), as well as for following the organ-acceptance after a kidney transplantation (Alvarez et al., 2013).

In 2007 the group led by Jan Löttvall found that EXs carry different functional RNA species (Valadi et al., 2007). Renal mRNA levels have been used in the past as prognostic markers for kidney diseases (Eikmans et al., 2003), but this required an invasive kidney biopsy. Analysis of the protein and RNA content of urinary EXs provides a non-invasive alternative for evaluating changes in renal gene and protein expression, and it has been found that several exosomal microRNAs isolated from urine are suitable markers for certain kidney diseases. One study showed that exosomal miR-145 and miR-130a levels were elevated in patients with diabetic nephropathy, while levels of miR-155 and miR-424 were down-regulated (Barutta et al., 2013). Further studies with a larger group of patients would be necessary to confirm these results.

Several miRNA markers of CKD/renal fibrosis have been identified. Levels of exosomal miR-29a, miR-29c, miR-200b, and miR-200c were down-regulated in patients with moderate-to-severe fibrosis (Lv et al., 2013), but not in cases with mild fibrosis. The same paper also demonstrates that miR-29c provides indicators of renal function and the histological degree of fibrosis, making it the most prominent candidate for a biomarker of CKD, while (Lv et al., 2014) showed that the exosomal mRNA level of CD2-associated protein (CD2AP) was down-regulated in CKD patients, and even more so in patients with a more severe disease. Both of these reports not only identified disease markers, but also demonstrated that the level of miRNA/mRNA present in urinary EXs provides an opportunity to define the progression of the disease.

Protocols for analyzing exosomes and isolating RNA from them still have to be optimized further in order to yield unbiased, reliable results. Furthermore, it still needs to be ascertained whether levels of exosomal mRNA can also reflect levels of the proteins used as biomarkers for kidney diseases. The same RNA and protein markers as were used in the past when a kidney biopsy was performed might not apply to exosomal RNA and protein levels, as is supported by the fact that little equivalence was found between the exosomal and cellular RNAs of exosome-producing cells (Skog et al., 2008; Mittelbrunn et al., 2011; Koppers-Lalic et al., 2014). This implies an underlying mechanism for the targeted loading of certain RNA species into EXs, in contrast to the loading of the most abundant cellular RNA into EXs in order to discard it. Future studies will have to elucidate the sorting mechanisms responsible for this process. Nonetheless, it is this fact that makes EXs especially interesting, as mRNAs and miRNAs can influence gene expression in the recipient cell and providing the EXs with their therapeutic potential.

One therapeutic strategy would be that RNA-bearing EXs would deliver their cargo to specific malfunctioning target cells and could restore damaged or deregulated protein production. Several studies have shown that exosomes shuttle functional miRNA and influence the gene expression levels of target cells (Pegtel et al., 2010; Montecalvo et al., 2012; Chen et al., 2014c).

Another promising feature of EXs is to serve as a non-cytotoxic drug delivery system. The challenge still lies in loading the drugs onto the EXs without imperiling their biological properties (Suntres et al., 2013). Different methods for loading a defined cargo onto EXs have been established, as recently reviewed (Johnsen et al., 2014). This is a possible way of developing novel therapeutics to treat various diseases. Some examples exist of studies which are currently in clinical trial phase I.

One such investigation concerns the potential application of EXs to deliver curcumin (which has proved to have biological activity) to colon cancer tissue (clinical trial no. NCT01294072),

while another uses EXs as a vaccine, administering dendritic cell-derived EXs (CSET 1437) loaded with antigen to lung cancer patients in order to activate their innate and adaptive immunity during therapy (clinical trial no. NCT01159288). Furthermore, the potential application of EXs to the treatment of kidney diseases is being explored. As described in Section The Role of Exosomes in Kidney Regeneration and Diseases above, various groups have shown that MSCs have a paracrine effect on acute and chronic kidney diseases (Gatti et al., 2011; van Balkom et al., 2011; Fang et al., 2013) in which soluble factors such as those associated with the secreted vesicles have a positive influence on cell behavior and promote the initiation of recovery. The potential of EXs for use in cell-free therapy applications has been summarized recently (Vishnubhatla et al., 2014) and will not be discussed here in detail. It is clear, however, that EXs from various cells such as MSCs and cancer cells have a great potential as novel therapeutic tools, and they can be expected to have a significant impact on the development of diagnostics and new treatments, for kidney diseases among others.

CONCLUSIONS

It has become generally accepted for the moment that EXs are present in a wealth of body fluids and are not only a cellular waste system as was thought earlier. Collectively, they may represent a newly identified but apparently ancient humoral system controlling homeostasis and disease, and they also provide a useful bank of biomarkers for a variety of diseases and may raise the value of urine as a non-invasive diagnostic component in medical practice. The analysis of urinary EXs not only provides us with prognostic disease markers, but might also make it possible to differentiate between diseases which display similar symptoms. Furthermore, certain data have shown that it is possible to estimate the severity of a disease, and hence its progression. This could enable non-invasive monitoring of responses to treatment and also make it possible to look for complications after kidney transplantations. The use of EXs as therapeutics and vaccines seems to be safe and effective due to their target specificity and their lack of cytotoxicity. This and the fact that a specific cargo can be loaded into them also make them very promising candidates as novel drug delivery systems. This is still something of a challenge, however, and better protocols need to be developed. More research will be necessary to ensure the safety of the resulting medical applications.

A schematic overview of the signaling role of EXs in relation to the kidney is presented in **Figure 2**. It is known that EXs play a role in kidney diseases and renal cancer, but many of the signaling molecules found during nephrogenesis occur in association with EXs, leading us to conclude that they not only participate in disease processes but also play a putative role in developmental processes. Which machinery regulates these processes during embryonic kidney development still remains largely an open

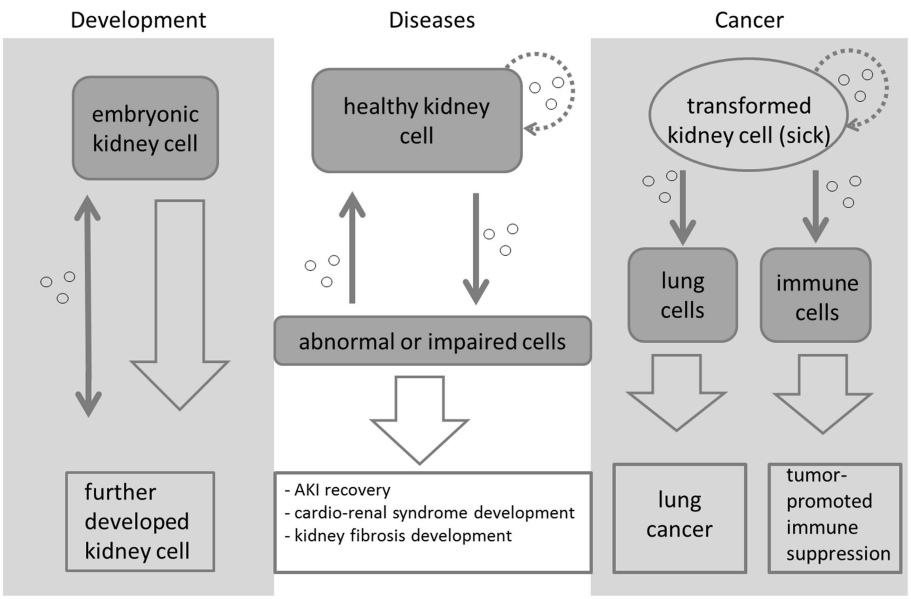

FIGURE 2 | Exosome-mediated signaling during kidney development, disease, and cancer. The signaling is symbolized by a thin gray arrow, while the development from one cell type/cell state to another is symbolized with a larger arrow. Different types of embryonic kidney cells release vesicles (small circles) to transfer signals during morphogenesis. These initiate and regulate the development of the organ. Exosomes are a means of cell-to-cell communication. When abnormal kidney conditions appear, exosomes can be released from the abnormal cells to be subsequently taken up by healthy cells, which will then be transformed and might even become abnormal as well. It has been reported that exosomes released from transformed kidney cells can initiate cancer in lung cells (Grange et al., 2011; Rana et al., 2012) and lead to tumor-promoted immune suppression in certain immune cells (Yang et al., 2013; Diao et al., 2015; Gu et al., 2015). Kidney cells and transformed kidney cells also take up the released vesicles again (dotted lined arrows). Most of the means of signaling depicted here are still not very well-characterized or understood.

question, but the accumulating evidence is starting to point toward a critical role for EXs in inductive signaling in the kidney. Future work should be targeted toward elucidating the normal physiological roles of EXs *in vivo* in defined disease models and advancing our knowledge of the precise mechanisms by which they promote renal diseases and cancer.

AUTHOR CONTRIBUTIONS

MK drafted the manuscript and prepared the figures and tables. AS helped with writing the manuscript and designing the figures. AS and SV critically reviewed and revised the manuscript. All three authors read and approved final version of the manuscript.

FUNDING

This work was supported by the Academy of Finland (206038, 121647, 250900, and 260056), Centre of Excellence grant 2012–2017 of the Academy of Finland (251314), the Sigrid Jusélius Foundation and the European Community's Seventh Framework Program, FP7/2009, under grant agreement 305608 (EURenOmics: European Consortium for High-Throughput Research in Rare Kidney Diseases).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 01 September 2015
doi: 10.3389/fcell.2015.00053

Developing a functional urinary bladder: a neuronal context

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The development of organs occurs in parallel with the formation of their nerve supply. The innervation of pelvic organs (lower urinary tract, hindgut, and sexual organs) is complex and we know remarkably little about the mechanisms that form these neural pathways. The goal of this short review is to use the urinary bladder as an example to stimulate interest in this question. The bladder requires a healthy mature nervous system to store urine and release it at behaviorally appropriate times. Understanding the mechanisms underlying the construction of these neural circuits is not only relevant to defining the basis of developmental problems but may also suggest strategies to restore connectivity and function following injury or disease in adults. The bladder nerve supply comprises multiple classes of sensory, and parasympathetic or sympathetic autonomic effector (motor) neurons. First, we define the developmental endpoint by describing this circuitry in adult rodents. Next we discuss the innervation of the developing bladder, identifying challenges posed by this area of research. Last we provide examples of genetically modified mice with bladder dysfunction and suggest potential neural contributors to this state.

Keywords: micturition, continence, pelvic ganglion, dorsal root ganglion, inferior hypogastric plexus, visceral afferent, neural development, organogenesis

OPEN ACCESS

Edited by:
Sunder Sims-Lucas, University of Pittsburgh, USA

Reviewed by:
Martin C. Michel, Boehringer Ingelheim Pharma GmbH & Co. KG, Germany
Kenneth A. Walker, University of Pittsburgh, USA

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 June 2015*
Accepted: *14 August 2015*
Published: *01 September 2015*

Citation:
Keast JR, Smith-Anttila CJA and Osborne PB (2015) Developing a functional urinary bladder: a neuronal context. Front. Cell Dev. Biol. 3:53. doi: 10.3389/fcell.2015.00053

Understanding the Endpoint: Innervation of the Adult Urinary Bladder

Overview of Bladder Innervation

The urinary bladder functions to collect and store urine so that it can be released at behaviorally appropriate times. These alternating phases of continence and micturition are precisely controlled by a hierarchical system of neural controls in the spinal cord and brain (Andersson and Wein, 2004; Beckel and Holstege, 2011; de Groat and Yoshimura, 2015). This circuitry connects with the bladder via a system of direct and indirect peripheral neural pathways summarized in **Figure 1**. In each component of this circuit, nerves involved in bladder control are intermingled with many other nerve types, as discussed in detail below.

This review will provide an overview of the features that govern the organization and function of bladder innervation in laboratory animals and humans. In general, the neural connectivity of the nerve supply to the bladder tissues is similar across species. However, it should be noted that while most contemporary studies use rodents, it was previously more common to use cats, which have larger nerves and are easier to study with neurophysiological techniques. Nevertheless, it is

Abbreviations: Ach, acetylcholine; DRG, dorsal root ganglion; NAd, noradrenaline (norepinephrine); NF200, neurofilament protein, 200 kd; TRPV1, transient receptor potential vanilloid receptor 1.

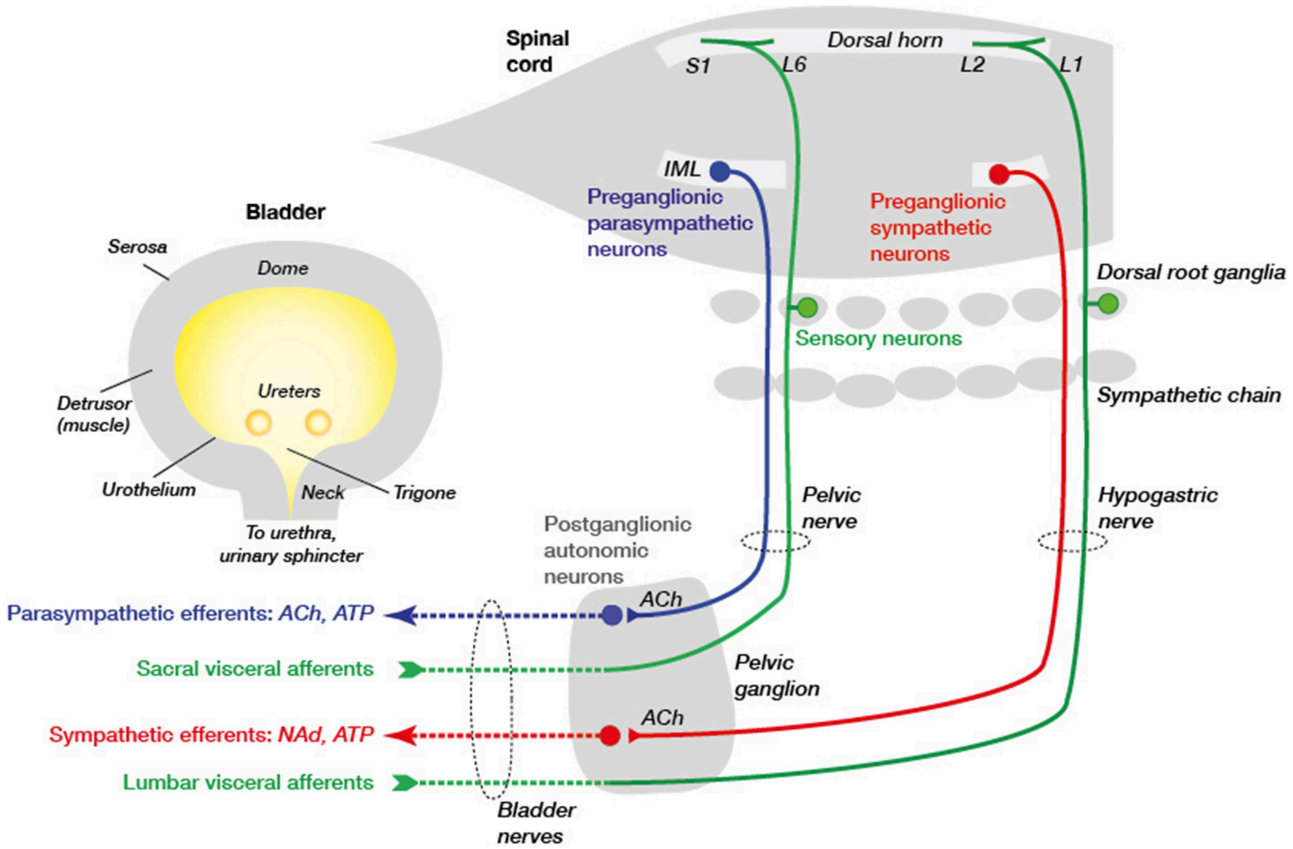

FIGURE 1 | Primary connections between the spinal cord, ganglia, and urinary bladder. Sensory neurons (green) in dorsal root ganglia (DRG) lie close to the spinal cord and make connections in the spinal cord and with the urinary bladder tissues. In rodents these DRG neurons are concentrated in spinal levels L1–L2 ("lumbar") and L6–S1 ("sacral"). They are activated by various physiological and pathophysiological changes within the bladder wall and signal to neurons in the spinal cord and, in turn, the brain. Integrative centers in the brainstem determine whether sympathetic (red) or parasympathetic (blue) pathways are activated to promote, respectively, continence or voiding. These pathways comprise two types of neurons, the first of which are preganglionic neurons in the intermediolateral nucleus (IML) of the spinal cord and the second are postganglionic neurons with cell bodies in the mixed pelvic ganglia. The preganglionic neurons release acetylcholine (ACh) to excite postganglionic neurons in pelvic ganglia that then release different transmitters to tissues in the bladder wall (ACh, NAd [noradrenaline] and ATP). Sympathetic preganglionic neurons also traverse the inferior mesenteric ganglion (not shown) before they reach the pelvic ganglion. Vascular innervation is not shown but is likely to arise from sympathetic chain rather than pelvic ganglion neurons. These autonomic motor pathways are coordinated with sphincter activity, mediated by somatic neurons in the spinal cord (not shown) that directly innervate the striated muscle of the external urethral sphincter. The axons from these neurons are not shown as they do not traverse the pelvic ganglion but project in the pudendal nerve.

clear that rodents have limitations for studying some specific questions relevant to human clinical urology, such as urethral sphincter control. As species differences cannot be discussed in detail here, the reader is referred to recent reviews that discuss this and other species differences in detail (Andersson and Wein, 2004; Thor and de Groat, 2010; Andersson et al., 2011).

Despite the paucity of data on the development of urogenital innervation and mechanisms of its remodeling in adulthood, the importance of this area of the nervous system is illustrated by the many clinical problems that arise when the nervous system is perturbed. For example, various types of pelvic surgery performed for tumor removal unavoidably damage nearby nerves, resulting in incontinence and sexual dysfunction (Mauroy et al., 2003; Cornu et al., 2014; Wit and Horenblas, 2014; Laterza et al., 2015). Understanding factors that drive growth of these connections during development could identify strategies for their repair in adults. Conversely, chronic cystitis is accompanied by bladder hyperinnervation (Dickson et al., 2006; Boudes et al., 2013) that may contribute to hyperactivity and pain. In this case, identifying growth inhibitory or chemorepulsive factors involved in bladder nerve development may provide targets for reducing aberrant growth and dysfunction in adults.

The nerves of the bladder can be divided into two classes—sensory and autonomic—that differ in their anatomy, chemistry, and function (Jänig, 2008). There are multiple subtypes of sensory and autonomic nerves, as discussed below. As the bladder fills, the bladder wall relaxes to accommodate increasing volume and sympathetic nerves constrict the bladder base and urethra to promote continence. In cats, sympathetic innervation of the detrusor may contribute to continence (Vaughan and Satchell, 1992; Khadra et al., 1995) including by modulating transmission in parasympathetic ganglion neurons

(de Groat and Saum, 1972), but this remains a debatable area in rodent research. Continence continues until a threshold tension of the bladder wall is detected by low-threshold mechanosensors (sensory neurons). This can trigger voiding but in healthy adults is dependent on the overriding neural controls from the brain. When suitable to initiate voiding, bladder contraction is activated in coordination with relaxation of the bladder base and urethra, and opening of the external urinary sphincter (striated muscle). This "voluntary" sphincter component of bladder control sets it apart from most other areas of autonomic function (e.g., cardiovascular regulation) that are activated in direct response to changing environmental and bodily needs but are "involuntary" (Jänig, 2008). The innervation of the external sphincter (urethral rhabdosphincter) originates from somatic motor neurons in the spinal cord and will not be discussed further here.

Sensory Innervation of the Bladder

Sensory axons form a rich network in most bladder tissues (Gabella, 1995, 1999; Gabella and Davis, 1998; Forrest et al., 2014). They distribute uniformly in the smooth muscle (detrusor) of the bladder wall, but near the urothelium become heavily concentrated at the base and neck—where some axons also penetrate the deeper layers of urothelial cells (Gabella and Davis, 1998; Gillespie et al., 2006; Forrest et al., 2014). This suggests signaling from the urothelium to sensory nerves, which is important in both health and disease (Kanai, 2011; Birder and Andersson, 2013), is spatially restricted. Sensory axons are also found within many blood vessels in the bladder wall (Gabella and Davis, 1998; Forrest et al., 2014) where their role is unknown.

As in other internal organs, sensory axons in the bladder terminate in simple, free endings (Gabella, 1995, 1999; Gabella and Davis, 1998; Forrest et al., 2014). Although often regarded as structurally unspecialized, different branching patterns and terminal structures occur but these variations are relatively subtle in comparison with the large, complex structures at the terminals of specialized sensory nerves in skin and skeletal muscle (e.g., Pacinian and Ruffini corpuscles). These small structural variations have not yet been correlated with particular sensory modalities in the bladder.

Sensory axons in the bladder originate from distant neuronal cell bodies in the lumbosacral dorsal root ganglia (DRG) that lie close to each side of the spinal cord (**Figure 1**). After leaving the DRG, their axons pass through the pelvic autonomic ganglia *en route* to the bladder, then intermingle with the bladder-projecting motor axons until they reach the bladder wall. Sensory and motor axons cannot be distinguished at this point unless immunolabeled for specific neural markers. As most bladder sensory axons originate from the sacral DRG these neurons are better characterized and more commonly experimentally manipulated than those originating from upper lumbar DRG (Kanai, 2011; Gonzalez et al., 2014; de Groat and Yoshimura, 2015). Given only a minority of neurons in DRG of both spinal levels project to the bladder (Robinson and Gebhart, 2008; de Groat and Yoshimura, 2009), assessing their molecular changes during disease or injury states raises a technical challenge. Anatomical tracing strategies [e.g., retrograde tracer micro-injection into the bladder wall Yoshimura et al., 2003; Forrest et al., 2013; Gonzalez et al., 2014] have been critical for visually separating bladder sensory neurons to define their properties.

Physiological studies recording electrical activity within single axons entering the bladder wall or at their terminals during different types of bladder manipulation (e.g., stretch) have sub-classified them by transduction properties (Zagorodnyuk et al., 2007, 2010; Xu and Gebhart, 2008). The predominant class acts as low-threshold mechanosensors that respond to tension and contraction. However, other classes of low-threshold and high-threshold mechano- and chemo-sensitive nerves can be recruited, especially in response to physical or chemical signals of impending bladder damage (Kanai, 2011; Skryma et al., 2011). These latter signals may be interpreted as sensations of discomfort or pain, but normally the organism does not perceive activity in these neural pathways.

Bladder sensory nerves are either lightly myelinated (A-delta) fibers or unmyelinated (C-fibers) (Kanai, 2011; de Groat and Yoshimura, 2015). Most of the A-delta class comprises low-threshold mechanosensors whereas most C-type fibers are higher threshold sensors that respond to diverse types of stimuli (polymodal), including damage. Measurements of action potential conduction velocity distinguish these two classes, but molecular properties are also useful indicators. For example, the majority of C-fiber bladder sensory neurons express the nociceptive transducer ion channel, TRPV1 (transient receptor potential vanilloid receptor 1), as well as the neuropeptides, calcitonin gene-related peptide and substance P (Arms and Vizzard, 2011; Skryma et al., 2011; Forrest et al., 2013). Functional studies have also used capsaicin-sensitivity to identify activity attributed to bladder sensory neurons that express TRPV1 (de Groat and Yoshimura, 2009; Michel and Igawa, 2015). Conversely, the 200 kD neurofilament protein (NF200) distinguishes the cell bodies of sensory neurons with myelinated axons (Lawson et al., 1993), including the A-delta bladder afferents (Forrest et al., 2013). However, the latter has limited use in the identifying their terminals where neurofilament levels are low (Forrest et al., 2014). Also, NF200 is not diagnostic of sensory axons in the bladder where many autonomic axons express this protein (Forrest et al., 2014).

Motor Innervation of the Bladder

The bladder can show myogenic activity that is independently driven by detrusor smooth muscle, but the effectors for the coordinated activity required by continence and micturition are parasympathetic and sympathetic autonomic neurons (Beckel and Holstege, 2011; de Groat and Yoshimura, 2015). Autonomic neurons also control vascular tone in the bladder (Michel and Igawa, 2015).

In the bladder, autonomic axons innervate the same tissues as the sensory axons, albeit in different density—in the detrusor and vasculature, autonomic axons are more prevalent than sensory axons, whereas the converse is true for the urothelium (Gabella and Davis, 1998; Gabella, 1999; Gillespie et al., 2006; Forrest et al., 2014). Although less prevalent than sensory axons, autonomic axons show a similar gradient of urothelial

innervation toward the bladder base and neck (Dickson et al., 2006).

Parasympathetic axons release acetylcholine as a transmitter and in some tissues and species release a co-transmitter, ATP (Jänig, 2008). This is the dominant axon type in the detrusor, where they cause muscle contraction during micturition (de Groat and Yoshimura, 2015). Sympathetic axons release noradrenaline (norepinephrine) and potentially also ATP but are scarce in the detrusor, only being found in small numbers in the base and neck of the bladder where they have a role in continence (Kihara and de Groat, 1997; Gosling et al., 1999; Forrest et al., 2014; de Groat and Yoshimura, 2015). A small population of parasympathetic axons lies close to the urothelium, where they are closely associated with sensory terminals (Dickson et al., 2006; Gillespie et al., 2006). The dominant population of sympathetic axons supplies the vasculature (Gabella, 1999; Forrest et al., 2014), where they likely cause constriction.

Most of the autonomic axons innervating the bladder originate from neuron cell bodies in the nearby pelvic ganglia (**Figure 1**) (Keast and de Groat, 1989). Sympathetic axons innervating the bladder vasculature most likely originate from neurons in the sympathetic chain (paravertebral) ganglia, as is the case for sympathetic vasoconstrictor axons in other organs (Jänig, 2008). The pelvic ganglia of rodents are functionally equivalent to the much more dispersed inferior hypogastric plexus in humans (Keast, 1999; Shoja et al., 2013). Rodent pelvic ganglia comprise paired clusters of thousands of neurons lying close to the uterine cervix and lateral lobes of the prostate, and contain many cell types. First, they contain neurons that each innervate a distinct region of the urogenital tract and lower bowel (Keast and de Groat, 1989; Kepper and Keast, 1995). Therefore, as discussed for identifying bladder sensory neurons in DRG, strategies such as retrograde tracing are valuable. Second, these target-defined neurons comprise both sympathetic and parasympathetic classes (Keast, 1995, 1999; Kanjhan et al., 2003; Jobling and Lim, 2008). Therefore, during development axons from two different regions of the spinal cord converge on the ganglia to synapse with the appropriate type of ganglion neuron. This mixture of sympathetic and parasympathetic neurons in one ganglion occurs nowhere else in the body. In addition to the pelvic ganglia, in rodents a few autonomic ganglion neurons are embedded in the bladder wall (Gillespie et al., 2006; Forrest et al., 2014), albeit are less common than intramural neurons in human bladder (Gilpin et al., 1983).

Development of Bladder Innervation

Sensory and autonomic axons innervating the bladder mostly originate from neuronal cell bodies that are some distance from the bladder tissues although some neuronal cell bodies appear transiently in the bladder wall during development and early postnatal life, with very few remaining by adulthood (Zvarova and Vizzard, 2005). Their properties more closely resemble autonomic than sensory neurons (Zvarova and Vizzard, 2005; Forrest et al., 2014) but their function is unknown. Therefore, to understand developmental mechanisms, it is necessary to identifying the relevant neuronal populations within DRG and pelvic ganglia. This includes defining the cues that promote growth and survival, determine guidance and appropriate synaptic targeting, and specify appropriate signaling properties (e.g., ion channel expression, transmitter synthesis). Progress in most of these areas remains limited. Bladder tissues express a typical array of neurotrophic factors (Vizzard, 2000; Vizzard et al., 2000; Gonzalez et al., 2014), and many neurotrophic factor receptors have been identified in neurons within the pelvic ganglia and bladder sensory neurons of the lumbosacral DRG (Murray et al., 2004; Hiltunen et al., 2005; Palma and Keast, 2006; Vizzard, 2006; Forrest et al., 2013; Gonzalez et al., 2014), but their roles in development of bladder innervation have not been defined.

Nerves grow into the bladder tissues well before birth. Axons enter the human fetal urinary bladder by 13 weeks post-conception and their density across each tissue type increases from that time (Kimmel and Mc, 1958; Klück, 1980; Gilpin et al., 1983; Dixon and Jen, 1995; Jen et al., 1995; Dixon et al., 1997, 1998, 1999). In these studies, immunohistochemistry for neuronal markers such as neuropeptides confirms that each of the major chemical classes of axons is likely to be present prior to birth. Moreover, neuronal cell bodies with transmitter chemistry similar to rodent pelvic ganglion neurons are embedded within the bladder wall and on its surface (Gilpin et al., 1983; Dixon et al., 1997); it is likely they are extensions of the inferior hypogastric plexus.

Our understanding of the timing and mechanisms of bladder innervation during embryonic development remains limited. In rodents, neural activation of the detrusor is evident just a few days after birth, but the properties of these contractions (including transmitter and receptor type) continue to change over the first couple of weeks (Levin et al., 1981; Maggi et al., 1984; Kruse and De Groat, 1990; Iuchi et al., 1994; Sann et al., 1997). Numerous images on the GUDMAP database (www.gudmap.org) (Harding et al., 2011) show that in embryonic mice, axons first reach the outer region of the detrusor by embryonic day (E) 14–15, and the urothelium by E18. At this time, axons can be distinguished by immunohistochemical markers that in adults would indicate parasympathetic, sympathetic and sensory axons. However, these may not be reliable markers in developing systems, where gene expression patterns may be quite dynamic. For example, tyrosine hydroxylase identifies adult noradrenergic sympathetic axons, but in development this enzyme is transiently expressed by a much broader neuron population (Rohrer, 2011). There is some early transient expression of cholinergic markers (Rohrer, 2011), and neuropeptide expression may be dynamic. Moreover, if expression of a particular neural marker is absent, it is tempting (but possibly inaccurate) to infer that the axon itself is not yet present.

Bladder-projecting sensory and autonomic neurons comprise only a minority of the neurons in pelvic ganglia and DRG, therefore studying their developmental mechanisms requires their identification (or separation) from surrounding neurons. Whereas, retrograde labeling can be conducted as simple recovery surgery in adult animals, comparable studies cannot be performed in the living embryo. Tracing nerve tracts *in vitro* by applying lipophilic dyes to fixed tissues (Ratcliffe et al., 2006) would be a valuable approach. This may also enable

topographic maps to be constructed for developing bladder-projecting neurons, as performed previously for different types of neurons in pelvic ganglia of adult rats (Keast, 1999). In addition to correlating with expression patterns of transmitters, intriguingly expression patterns of transcription factors have been reported in developing mouse pelvic ganglia (Wiese et al., 2012), some of which may correlate with bladder-specific pathways. In parallel with the order of organ maturation, is also possible that bladder-projecting pelvic ganglion neurons differentiate and mature much earlier than those innervating reproductive organs. Therefore, to determine the mechanism by which the bladder becomes innervated, each target tissue within the bladder must be examined, a range of ganglion systems investigated and the neurons within those ganglia that project to bladder identified. Immunohistochemical tools enable distinction of some elements within this system, but without a specific molecular phenotype yet identified for bladder-projecting neurons, progress in defining the route and source of connections will be constrained. Understanding development of mechanisms by which nerves communicate with non-neuronal cells within the bladder, such as glial cells and interstitial cells (Gabella, 1995, 1999; McCloskey, 2011) is also important.

Genetic Models of Bladder Dysfunction

Animal models provide valuable insights into the factors contributing to particular bladder phenotypes and the mechanisms driving development of normal bladder innervation. Here we highlight some examples of mouse models with dysfunction due to enlarged bladders. We will discuss these in the context of possible neuronal impairment derived from loss of sensory transduction (to detect distension) or motor control (to enable contraction and emptying).

Deletion of the nicotinic acetylcholine receptor Chrna3, or the combined deletion of Chrnb2 and Chrna4, result in a bladder phenotype known as megacystis (Xu et al., 1999a,b). This is characterized by extreme bladder enlargement, overflow incontinence and bladder infection with urinary stones. These features are likely driven by neuronal dysfunction, as nicotinic receptors are expressed by autonomic ganglion neurons and required for transmitting the excitatory message from spinal neurons to ganglion cells, and hence to the organs. Specifically, Chrna3 and Chrnb4 are highly expressed in pelvic ganglia (Park et al., 2006; Girard et al., 2013) and are upregulated in parasympathetic pelvic ganglion neurons in a surgical obstruction model of the urethra (Chung et al., 2015). While not excluding other mechanisms, these reports are consistent with the bladder dysfunction being driven by an inability to void, resulting in distension and hypertrophy of the bladder. This model could provide additional insights into the impact of autonomic inactivity (rather than neuronal loss) on upstream components of the reflex circuitry. The effects of prolonged distension on sensory neurons could reveal mechanisms relevant to a number of clinical obstructive conditions.

A more bladder-specific disruption has been seen with "megabladder" mice (Singh et al., 2007; McHugh, 2014), a phenotype caused by randomly inserting a transgene into chromosome 16 that subsequently, along with a portion of chromosome 16, translocated to chromosome 11 downstream of myocardin. Profound changes, including severe distension and thinning of bladder wall were reported *in utero*, with the bladder almost completely lacking detrusor muscle; less dramatic changes occur in lamina propria and urothelium. This severe problem was restricted to lower urinary tract; muscle development was normal in the gastrointestinal and respiratory tracts, and vascular system. The innervation of these bladder tissues has not yet been examined but could reveal if and how a normal detrusor determines normal bladder innervation patterns. Furthermore, the phenotype was more severe in male, providing a platform to study sex differences in neuronal development.

A different outcome occurred after deleting FGFR2 (fibroblast growth factor receptor 2) from the bladder mesenchyme, leading to thinner detrusor muscle but in this case a thickened, collagen-enriched lamina propria (Walker et al., 2015). This impacted on muscle contractility in response to cholinoceptor and purinoceptor agonists and compliance of the bladder wall. Innervation density was not examined, but their decreased intervoiding interval in cystometry studies provides further encouragement to examine sensory nerve density and threshold for activation in these animals.

Conclusions

This review highlights the primary features of bladder innervation in the adult in order to demonstrate the gaps to be filled in understanding the development of this anatomically and functionally complex nerve supply. Defining the developmental mechanisms in this system may also reveal strategies to drive regrowth, targeting and functional recovery of bladder nerves in the adult.

Acknowledgments

This work was supported by the National Institutes of Health (5U01 DK094479 to JK) and the National Health and Medical Research Council of Australia (1003512 to PO and JK, 1022941 to JK).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 13 January 2016
doi: 10.3389/fcell.2015.00085

Interleukin-22 Signaling in the Regulation of Intestinal Health and Disease

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Interleukin (IL)-22 is a member of the IL-10 family of cytokines that has been extensively studied since its discovery in 2000. This review article aims to describe the cellular sources and signaling pathways of this cytokine as well as the functions of IL-22 in the intestine. In addition, this article describes the roles of IL-22 in the pathogenesis of several gastrointestinal diseases, including inhibition of inflammation and barrier defense against pathogens within the intestine. Since many of the functions of IL-22 in the intestine are incompletely understood, this review is meant to assess our current understanding of the roles of IL-22 and provide new opportunities for inquiry to improve human intestinal health and disease.

Keywords: interleukin-22, gastrointestinal tract, epithelial cells, barrier defense

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *14 August 2015*
Accepted: *14 December 2015*
Published: *13 January 2016*

Citation:
Parks OB, Pociask DA, Hodzic Z, Kolls JK and Good M (2016) Interleukin-22 Signaling in the Regulation of Intestinal Health and Disease. Front. Cell Dev. Biol. 3:85. doi: 10.3389/fcell.2015.00085

INTRODUCTION

Since its discovery in 2000 (Dumoutier et al., 2000a), interleukin-22 (IL-22) has been widely studied for its diverse roles in cell proliferation, tissue regeneration, cellular defense, and inflammation. IL-22 is expressed by inflammatory cells in a number of tissues in the body including the lungs, liver, kidneys, thymus, pancreas, breast, gut, skin, and synovium, reviewed in Dudakov et al. (2015). The focus of this review article is on the distinct roles of IL-22 within the intestine. One of the main functions of IL-22 is to support and maintain the gastrointestinal (GI) epithelial barrier as well as to facilitate barrier defense mechanisms against bacterial pathogens such as *Clostridium difficile* (Hasegawa et al., 2014), *Citrobacter rodentium* (Zheng et al., 2008; Muñoz et al., 2015), and *Toxoplasma gondii* (Muñoz et al., 2015). Studies have shown that regulation of IL-22 is an important component of many diseases including graft-versus-host disease (GVHD) (Munneke et al., 2014), inflammatory bowel disease (IBD), specifically Crohn's disease (Wolk et al., 2007; Schmechel et al., 2008; Souza et al., 2013), ulcerative colitis and the experimental model of ulcerative colitis dextran sodium sulfate (DSS)-induced colitis (Sugimoto et al., 2008) as well as acute polymicrobial sepsis (Weber et al., 2007). The diverse effects of IL-22 against several inflammatory conditions implicate IL-22 as a promising therapeutic target for these types of GI-related illnesses. The purpose of this review article is to describe the distinct roles of IL-22 in the regulation of health and disease in the intestine.

CELLULAR SOURCES OF IL-22

IL-22 is a member of the IL-10 family and has recently been grouped into a smaller subset of cytokines called the IL-20 subfamily, which is comprised of IL-19, IL-20, IL-22, IL-24, and IL-26 (Ouyang et al., 2011; Rutz et al., 2014). This family was identified based on their cellular targets and ability to recognize similar receptor subunits.

Many different types of immune cells produce IL-22. In lymphoid tissues, αβ and γδ T cells, innate lymphoid cells (ILCs; Cella et al., 2009; Colonna, 2009; Cella et al., 2010; Sonnenberg et al., 2011a; Hanash et al., 2012; Lee et al., 2012; Spits et al., 2013; Korn et al., 2014) and NK T cells (NKTs) all produce IL-22. In addition to the IL-22 produced in lymphoid tissue, macrophages, neutrophils (Zindl et al., 2013; Lee et al., 2015), dendritic cells (Mann et al., 2014), and fibroblasts (Ikeuchi et al., 2005) have also been identified as sources for IL-22; however, these non-lymphoid sources produce less IL-22 relative to the lymphoid sources. Depending on the tissue type, other non-traditional cell types can produce IL-22. For example, in the gastrointestinal tract of mice with experimental colitis, neutrophils become activated and are subsequently capable of expressing IL-22 (Zindl et al., 2013; Lee et al., 2015). In a mouse model of DSS-induced colitis, Zindl et al. demonstrated that depletion of Ly6G/C$^+$ neutrophils/dendritic cells using RB6-8C antibody resulted in reduced colonic levels of IL-22, and additionally, these isolated colonic neutrophils were capable of producing IL-22 after IL-23 stimulation (Zindl et al., 2013). In a mouse model of infectious colitis, Lee and colleagues found the CD11b$^+$ Ly6C$^+$ Ly6G$^+$ subset of neutrophils were the main source of IL-22 secretion (Lee et al., 2015). This type of non-traditional expression of IL-22 can be found in patients with diseases outside the GI tract such as rheumatoid arthritis (RA), where fibroblasts have been found to contribute to the production of IL-22 (Ikeuchi et al., 2005). Accordingly, cellular sources of IL-22 are numerous, and the main production sites of IL-22 in lymphoid-derived cells are described below:

αβ T Cells

IL-22 production has been demonstrated by three subtypes of T helper (Th) cells: Th1, Th17, and Th22. The prototypical IFN-γ producing Th1 cells can produce IL-22 without additional growth factors during effector function. However, naive T cells require the presence of transforming growth factor-β (TGF-β) to drive either the formation of Th17 cells, which can then produce IL-22, and/or T regulatory cells (Tregs; Littman and Rudensky, 2010). The cytokine IL-6 drives the expression of retinoic acid-related orphan receptor-γt (RORγt) and IL-23, both of which are critical for Th17 cells to produce IL-22 (Zhou et al., 2007). The regulation of Th17 cells is further controlled by changes in the concentration of TGF-β (Zhou et al., 2008). When the concentration of TGF-β is low, Th17 cells are induced via IL-23-stabilization of RORγt (Zhou et al., 2008). However, when TGF-β is present in high concentrations, expression of the IL-23 receptor is inhibited, reducing the expression of both RORγt and IL-22, while also activating the transcription factor forkhead box P3 (FoxP3; Zhou et al., 2008). This leads to the expansion of Tregs. Thus, high concentration of TGF-β promotes Treg expansion while preventing further expansion of Th17 cells (Zhou et al., 2008). This TGF-β dependent suppression of IL-22 was found to be mediated by c-Maf, a transcription factor that binds to the IL-22 promoter (Rutz et al., 2011). Furthermore, IL-23 can counteract the effects of high concentrations of TGF-β by inducing production of IL-22 (Zhou et al., 2008).

Th22 cells also produce IL-22 and were first identified in the skin as a helper T cell population expressing CCR6, CCR4, and CCR10 (Duhen et al., 2009; Trifari et al., 2009). Th22 cells are classified as a subset of CD4$^+$ helper T cells characterized for their production of IL-22, IL-13, and tumor necrosis factor (TNF)-α, but not IL-17, IFN-γ, or IL-4 (Duhen et al., 2009). Th22 cells are further described by their inability to produce T-bet (a transcription factor known to control IFN-γ) and negligible expression of RORγt (a Th17 and IL-22 transcription factor; Duhen et al., 2009). However, the development of CD4+ T cells capable of producing IL-22 is dependent on the aryl hydrocarbon receptor (AhR) and T-bet (Basu et al., 2012). Moreover, a recent study has demonstrated that activation of signal transducer and activator of transcription factor 3 (STAT3) was required for IL-22 production by Th22 cells and was responsible for effective host clearance of infectious colitis (Backert et al., 2014).

γδ T Cells

γδ T cells are found in the intraepithelial lymphocyte (IEL) compartment of the intestine and secrete a variety of cytokines, including a significant amount of IL-22 (Sutton et al., 2009). γδ and αβ T cells share two features in common: (1) expression of RORγt and (2) expression of IL-22 in the presence of IL-23 (Martin et al., 2009; Sutton et al., 2009; Mabuchi et al., 2011; Mielke et al., 2013). Certain types of γδ T cells express Toll-like receptors (TLRs) and can directly interact with specific pathogen products (Martin et al., 2009; Crellin et al., 2010).

γδ T cells have roles in limiting the translocation of pathogens such as *Salmonella typhimurium* and *T. gondii* in the intestinal epithelial tissue (Edelblum et al., 2015). The γδ IELs contribute to the regulation and maintenance of gut homeostasis (Fuell et al., 2015). Studies have shown that mice deficient in γδ IELs (T Cell Receptor $\delta^{-/-}$ mice) have structural differences within their intestine compared to wild-type littermates, including altered intestinal glycosylation and glycan antennae (Fuell et al., 2015). These structural differences can contribute to a lack of mucosal protection in the gut of mice deficient in γδ IELs, indicating γδ IELs and γδ T cells play a role in intestinal host defense (Edelblum et al., 2015; Fuell et al., 2015).

Innate Lymphoid Cells (ILCs)

ILCs contribute to the innate and adaptive immunity of the intestine and can be found at mucosal surfaces or in cryptopatches, which are found in the lamina propria of the small intestine beneath the intestinal crypts (Diefenbach, 2012). ILCs are characterized by their lymphoid morphology, absence of cytotoxic capacity, and lack of B or T cell receptors as reviewed in Spits and Cupedo (2012) and Spits et al. (2013). ILCs are a relatively recent addition to the immune cell family and are grouped based on their functional characteristics and expression of cytokines and transcription factors (Spits et al., 2013). Group 1 ILC (ILC1s), defined by their expression of the transcription

factor T-bet, produce the Th1 cytokine IFN-γ and are critical to host response of intracellular infections (Klose et al., 2014). Group 2 ILCs (ILC2s) are dependent on the transcription factor GATA3 to produce the Th2 cytokines IL-5 and IL-13 (Hoyler et al., 2012) and are important in helminth infection (Fallon et al., 2006). Group 3 ILC (ILC3s) are defined by their expression of RORγt, production of the Th17 cytokines IL-17 and IL-22, and involvement in defense against extracellular bacterial or fungal infections (Sonnenberg et al., 2011b; Gladiator et al., 2013; Edelblum et al., 2015) as described in **Table 1** (Eberl et al., 2015).

Moreover, $CD4^+$ T cells can regulate ILC production of IL-22 as well as downstream production of antimicrobial peptides (AMPs) in an IFN-γ dependent manner (Korn et al., 2014). The AMPs evaluated in this study were regenerating islet derived 3 (Reg3) γ and β, members of the C-type lectin family (Gallo and Hooper, 2012), which are induced by IL-22 (Kolls et al., 2008; Zheng et al., 2008). Reg3β has been reported to kill *Escherichia coli* and may be involved in creating a niche of invading pathogens, specifically *S. typhimurium* (Stelter et al., 2011). In contrast, the related protein Reg3γ, which binds to peptidoglycan, has bactericidal activity against Gram-positive bacteria (Cash et al., 2006) and maintains a physical space between luminal bacteria and the epithelium (Vaishnava et al., 2011).

ILC3s can be found in the small and large intestinal mucosa, Peyer's patches and gut-associated lymphoid tissue (GALT; Cella et al., 2014). ILC3s include lymphoid tissue-inducer (LTi) cells and ILC3s that can be activated to express IL-22 by IL-23 through natural cytotoxicity receptor $(NCR)^+$ and NCR^- ILC3s (Spits and Cupedo, 2012; Spits et al., 2013). In both humans and mice, NCR^+ ILC3s do not produce IFN-γ, which promote NK cell function, differing these cells from typical NK cells (Colonna, 2009; Cella et al., 2010; Spits et al., 2013). In addition, IL-15 and RORγt are essential for the development and maturation of NCR^+ ILC3s (Satoh-Takayama et al., 2008; Cella et al., 2009; Luci et al., 2009; Sanos et al., 2009).

TABLE 1 | Description of the different types of innate lymphoid cells (ILCs).

Innate Lymphoid Cells				
Cell Type	**Immunologic function**	**Stimulation**	**Transcription factors**	**Effector cytokines**
ILC1	Intestinal inflammation	IL-12 IL-15 IL-18	T-bet	IFN-γ TNF-α
ILC2	Airway inflammation Helminth infection	IL-25 IL-33 TSLP	RORα GATA3 Bcl11b	IL-4 IL-5 IL-13
ILC3	Intestinal inflammation Gut barrier protection Lymphoid tissue development	IL-23 IL-1β	RORγt AhR	IL-17 IL-22 LT-$\alpha_1\beta_2$ GM-CSF

Natural Killer T Cells

All Natural Killer T (NKT) cells develop and mature in the thymus where they diverge from other cell types at the $CD4^+CD8^+$ double positive thymocyte stage (Mebius et al., 2001; Bendelac et al., 2007; Possot et al., 2011). Before this thymocyte stage, the αβ T cell receptor (TCR) of undifferentiated cells must recombine to bind to a CD1d molecule to progress developmentally (Bendelac et al., 2007). The NKT cell developmental cycle has unique stages of development (Bendelac et al., 2007; Godfrey et al., 2010) based on the NKT cells expression of CD24, CD44, or NK1.1 as well as the transcription factors PLZF, c-Myc, Egr2, RelA, or T-bet (Bendelac et al., 2007; Godfrey et al., 2010). NKT cells that produce IL-22 are also known to express CCR6, IL-23R, RORγt, paralleling γδ T cells, Th17 cells, and ILC3s (Bendelac et al., 2007; Godfrey et al., 2010). In addition, NKT cells produce IL-22 in the presence of IL-23 (Rachitskaya et al., 2008; Doisne et al., 2011; Moreira-Teixeira et al., 2011; Paget et al., 2012). Accordingly, NKT cells are similar to ILCs and γδ T cells in that these cell types can produce IL-22 without transcriptional activator interferon regulatory factor 4 (IRF4) signaling, whereas αβ T cells require IRF4 signaling to initiate IL-22 expression (Raifer et al., 2012). However, IL-22-producing NKT cells have been found to require interaction from TCR-CD1d to induce production of IL-22 (Doisne et al., 2011). Many details surrounding NKT cells that produce IL-22 are not well understood. A study has suggested that a stage during NKT cell development called "stage 0" ($CD24^+CD44^{lo}NK1.1^-$) is crucial to the maturation of these cells and is hypothesized to be controlled by RORγt (Benlagha et al., 2005); however, further studies are needed to completely understand the role of NKT cells in IL-22 production.

POSITIVE AND NEGATIVE REGULATORS OF IL-22

IL-22 is mainly produced by Th17 cells, ILCs, γδ T cells, and NKT cells (Ouyang et al., 2011). Th17 cells produce IL-22 in response to IL-6 and TNF-α in the setting of inflammation and trauma (Liang et al., 2006; Zhang et al., 2011). However, IL-6 can independently initiate the expression of IL-22 (Liang et al., 2006; Zheng et al., 2007). *Il-22* gene expression, initially found only in the thymus and brain (Dumoutier et al., 2000a,b; Sabat et al., 2014), has been discovered in the gastrointestinal tract, liver, lung, skin, pancreas, and spleen (Sabat et al., 2014). IL-22 expression and production is positively and negatively regulated by several molecules including IL-23, IL-7, the Notch signaling pathway, the AhR, IL-22 binding protein (IL-22BP), IL-25, and IL-1β. Our continued study of these molecules can aid in the understanding IL-22, particularly in the setting of gastrointestinal disease.

Interleukin-23

IL-23 is one of the main inducers of the expression and production of IL-22 (Kastelein et al., 2007). During differentiation of Th17 cells, IL-23 enhances IL-22 expression, which leads to increased expression of the IL-23 Receptor (IL-23R). This results in enhanced interaction between IL-23 and its receptor, and consequently, increased IL-22 production.

Activated dendritic cells (DCs) and macrophages, in response to microbial stimulation, are important sources of IL-23 (Langrish et al., 2004). IL-23 production by DCs is regulated by the lymphotoxin-β receptor (LTβR; Tumanov et al., 2011). IL-23 production by activated DCs via LTBR indirectly results in increased IL-22 by RORyt+ ILCs (Tumanov et al., 2011).

However, there are other cellular sources of IL-23. DSS-induced epithelial injury results in activation of LTβR signaling, which promotes intestinal mucosal healing by triggering production of IL-23 from intestinal epithelial cells (Macho-Fernandez et al., 2015). This ultimately results in increased IL-22 production by RORyt+ ILC3s, particularly CCR6+Tbet- CD4- and CD4+ LTi cells (Macho-Fernandez et al., 2015).

Interleukin-7

IL-7, a critical cytokine for the maintenance, development, and proliferation of αβ and γδ T cells, also acts to positively regulate the production of IL-22 (Cella et al., 2010). It is unlikely that the cytokine IL-7 directly regulates the expression of IL-22; however, IL-7 is necessary for the stable expression of RORγt, a transcription factor important in IL-22 expression (Vonarbourg et al., 2010). RORγt expression regulates the differentiation of cells that produce IL-22 to achieve the optimal conditions for the production of IL-22 (Nurieva et al., 2007; Qiu et al., 2012). These observations would suggest that IL-7 acts in an expansion role to promote the expression of IL-22 by many types of cells. However, IL-7 is not *directly* required for functional IL-22 cytokine to be produced in tissues (Peschon et al., 1994; von Freeden-Jeffry et al., 1995; Nurieva et al., 2007; Vonarbourg et al., 2010; Qiu et al., 2012).

Notch

Notch-induced stimulation of $CD4^+$ T cells increases the production of IL-22 within the intestine, which is important for epithelial cell proliferation and differentiation (Murano et al., 2014). Overexpression of Hes1, a Notch target gene, enhanced IL-22-induced STAT3 expression in a human intestinal epithelial cell line (Murano et al., 2014). However, in Notch-deficient mice, IL-22 signaling and production was eliminated (Alam et al., 2010; Murano et al., 2014). Lee et al. demonstrated that the Notch receptor was induced by the AhR (Lee et al., 2012), which promotes the development of ILCs and Th17 cells, ultimately leading to increased IL-22 production.

Aryl Hydrocarbon Receptor (AhR)

The AhR induces IL-22 in one of two ways. AhR can either (1) directly regulate IL-22 gene expression and cytokine production or (2) regulate the production and development of ILC3 and Th17 cells (Veldhoen et al., 2008; Lee et al., 2012; Qiu et al., 2012). AhR is found in the cytoplasm complexed with heat shock protein 90 (Hsp90; Esser et al., 2009), and once activated, the AhR complex can translocate to the nucleus where AhR can act as a transcription factor (Tsuji et al., 2014). Various ligands, physical stress, cyclic AMP, and calcium (Ca^{2+}) can all activate AhR. It is thought that AhR ligands derived from gut microbiota are not required for the development of ILCs (Veldhoen et al., 2008; Esser et al., 2009; Qiu et al., 2012). However, these ligands are thought to be an essential component to initiate the transcription of IL-22 (Denison and Nagy, 2003; Oesch-Bartlomowicz et al., 2005; McMillan and Bradfield, 2007; Nguyen and Bradfield, 2008; Puga et al., 2009; Lee et al., 2012; Zelante et al., 2013; Lowe et al., 2014).

Interleukin-1β

IL-1β is an influential cytokine, capable of activating NKT cells, ILC3s, and Th17 cells to produce IL-22 (Sutton et al., 2009; Doisne et al., 2011; Paget et al., 2012; Chen et al., 2013; Lee et al., 2013; Monteiro et al., 2013). Macrophages, DCs, neutrophils, B and T cells, endothelial cells, and epithelial cells all produce IL-1β, making this ligand a diverse molecule in cellular pathways (Sims and Smith, 2010). IL-1β production sustains expression of IL-22 *in vitro*, promotes the expansion of NK cells, specifically, human stage three immature NK cells, in conjunction with IL-15 from secondary lymphoid tissue DCs, and inhibits these NK cells from differentiating into IFN-γ-producing cells (Sutton et al., 2009). Of note, in IL-1 $receptor^{hi}$ immature NK cells, IL-1β must be *constantly* present in order for continual expression of IL-22. This is in direct contrast to IL-23, which only needs to be present to *initiate* the IL-22 signaling pathway so that IL-22 production continues in the absence of IL-23 (Hughes et al., 2010).

Negative Regulation of IL-22

There are several molecules that negatively regulate IL-22 expression. IL-22 Binding Protein (IL-22BP, also known as IL-22RA2) is a soluble receptor that is able to regulate IL-22 bioactivity, which has a 1000 times higher binding affinity for IL-22 compared to the IL-22 receptor (IL-22RA1) complex (Weiss et al., 2004; Wolk et al., 2007). Epithelial expression of IL-25 is able to repress IL-22 production by RORyt+ ILCs (Weiss et al., 2004; Wolk et al., 2007). Transforming growth factor-β (TGF-β) has several functions, specifically with respect to Th17 cells, RORγt, IL-23R, and IL-22 (Mangan et al., 2006; Zhou et al., 2007; Morishima et al., 2009). TGF-β is essential for the differentiation of Th17 cells and influences RORγt and IL-23R expression in various tissues (Morishima et al., 2009). In IL-22 signaling, TGF-β acts in a dose-dependent fashion to regulate the expression of IL-22 (Zheng et al., 2007; Volpe et al., 2009; Rutz et al., 2011; Penel-Sotirakis et al., 2012). However, the cytokine IL-23 is able to overcome the effects of TGF-β on IL-22, thereby increasing production of IL-22 (Volpe et al., 2009; Rutz et al., 2011). Furthermore, both the transcription factor c-Maf (an inhibitor of TGF-β, IL-22, IL-27) and the inducible costimulator (ICOS) pathways can influence IL-22 production (Bauquet et al., 2009; Paulos et al., 2010; Rutz et al., 2011). A recently described and poorly understood cytokine member of the IL-1 family, IL-38, can also regulate IL-22 production. At low concentrations, IL-38 can prevent the production of IL-22, while at high concentrations, IL-38 promotes the production of IL-22 (Tortola et al., 2012; van de Veerdonk et al., 2012). However, further studies are required to determine the signaling pathways involved with IL-38 and their biologic effects.

IL-22 Binding Protein

IL-22RA2, alternatively called IL-22BP, has been found throughout the body. It is a soluble secreted receptor with a binding structure similar to the membrane bound IL-22RA1

(Dumoutier et al., 2001; Wu et al., 2008). This structural homology allows IL-22BP to bind to IL-22, thereby inhibiting the binding of IL-22 to its receptor complex and consequently preventing IL-22 signaling (Wu et al., 2008). Interestingly, it has been observed that IL-22BP levels decrease with significant increases in IL-22 (Weiss et al., 2004; Wolk et al., 2007). IL-22BP levels increase only after persistently high levels of IL-22, indicating that IL-22BP has a regulatory role after the initial effects of the elevated IL-22 levels have been established (Wolk et al., 2007; Sugimoto et al., 2008; Huber et al., 2012). In the intestine, IL-22BP is highly expressed in colonic dendritic cells (Huber et al., 2012). When tissue damage in the intestine is detected, the NLRP3 or NLRP6 inflammasomes down-regulate IL-22BP via activation of IL-18 (Huber et al., 2012). Additionally, Martin et al. discovered a subset of conventional dendritic cells, lamina propria $CD103^{+}CD11b^{+}$ DCs, in both lymphoid and non-lymphoid tissues as a significant source of IL-22BP (Martin et al., 2014). Additionally, eosinophils within the human intestine were also identified as an important source of IL-22BP (Martin et al., 2015).

IL-22 RECEPTOR AND SIGNALING

The IL-22 receptor is a type 2 cytokine receptor comprised of the heterodimeric complex with IL-22RA1 and IL-10R2 (IL-10Rβ; Kotenko et al., 1997, 2001; Xie et al., 2000; Dumoutier et al., 2000c; Li et al., 2004). While IL-10R2 is constitutively expressed in cells throughout the body, IL-22RA1 is expressed almost exclusively in epithelial tissues (Wolk et al., 2004). Due to this specificity of IL-22RA1, it is hypothesized that this receptor has a defining role in facilitating the innate immunity of epithelial cells (Zheng et al., 2007, 2008). Interestingly, IL-22 has been shown to have no affinity for IL-10R2 and rather a very high affinity to bind to IL-22RA1 (Logsdon et al., 2002, 2004; Li et al., 2004; Wolk et al., 2004; Jones et al., 2008; Yoon et al., 2010). Binding of IL-22 to IL-22RA1 increases its affinity for IL-10R2 (Logsdon et al., 2002; Li et al., 2004; Logsdon et al., 2004; Wolk et al., 2004; Jones et al., 2008; Yoon et al., 2010), suggesting a stepwise process in the binding (Bleicher et al., 2008).

IL-22 signals through the IL-22 receptor complex, leading to activation of Janus kinase 1 (Jak1) and non-receptor protein tyrosine kinase 2 (Tyk2; Lejeune et al., 2002). This primarily leads to tyrosine residue phosphorylation of STAT3; however, STAT1 and STAT5 have also been shown to be activated by IL-22 (Lejeune et al., 2002). STAT3 activation in the intestinal epithelium is responsible for immune homeostasis as well as wound healing in an IL-22 dependent manner (Pickert et al., 2009). Several additional pathways are involved in IL-22 signaling, including Mitogen Activation Protein Kinase (MAPK), Akt (Sekikawa et al., 2010), and p38 pathways (Andoh et al., 2005).

IL-22 REGULATION OF HEALTH AND DISEASE: GASTROINTESTINAL TRACT

IL-22 has several roles in the gastrointestinal tract, including tissue regeneration and cell proliferation, defense against pathogens, as well as maintenance and protection of the intestinal barrier. Expression of IL-22 has been identified in the many tissues including the upper GI tract, the oral cavity, salivary glands, tonsils, stomach, and esophagus (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009; Ciccia et al., 2012; Delsing et al., 2012; Kato-Kogoe et al., 2012; Naher et al., 2012; Zhuang et al., 2012). In these tissues, IL-22 induces the production of defensins, Reg family molecules, and S100 proteins—all of which are innate antimicrobial molecules (Wolk et al., 2004, 2006; Liang et al., 2006; Zheng et al., 2008; Pickert et al., 2009; Sanos et al., 2011; Sonnenberg et al., 2012). These antimicrobials assist in providing gut barrier protection against pathogens (Sugimoto et al., 2008). During colitis, IL-22 is responsible for regulating mucin production from goblet cells, which constitutes the protective mucous layer that lines the intestinal epithelium (Sugimoto et al., 2008). However, further studies are needed to determine whether the intestinal mucin production is directly or indirectly related to goblet cells influenced by IL-22 expression.

Recent studies have demonstrated that patients with IBD, specifically Crohn's disease or ulcerative colitis have increased IL-22 expression in the colonic tissue (Hanash et al., 2012). Wolk et al. found systemically elevated IL-22 in Crohn's disease and intestinal elevation of IL-22 in a mouse model of colitis (Wolk et al., 2007). In both circumstances, LPS-binding protein (LBP) was also found to be upregulated in the blood (Wolk et al., 2007). Moreover, administration of IL-22 to healthy mice resulted in increased LBP at concentrations capable of neutralizing LPS, and consequently, inflammation. This suggests that IL-22 can act as an anti-inflammatory molecule against LPS and may be a novel therapeutic agent in IBD patients (Witte et al., 2010; Sonnenberg et al., 2011a; Sabat et al., 2014).

In patients with IBD, there is significantly decreased expression of AhR in intestinal tissue, and in response to AhR agonist, 6-formylindolo(3, 2-b) carbazole (Ficz), isolated intestinal lamina propria mononuclear cells in this patient population resulted in increased IL-22 (Monteleone et al., 2011). Moreover, in multiple experimental mouse models of colitis, administration of Ficz subsequently caused IL-22 induction, whereas administration of an AhR antagonist resulted in more severe colitis with decreased IL-22 production (Monteleone et al., 2011).

IL-22 has a critical role in antibacterial immunity and host defense in the intestine. IL-22 is important in the clearance of the mouse pathogen *C. rodentium*, which causes infectious colitis that mimics *E. coli* infection in humans (Zheng et al., 2008; Sonnenberg et al., 2011b). Furthermore, infectious colitis with *C. rodentium* can be suppressed with ILC production of IL-22 (Qiu et al., 2012) in an AhR and microbiota-dependent manner (Qiu et al., 2013). This is in accordance with the observation that IL-22 is elevated in the colon in response to infection by *C. difficile* or *C. rodentium* (Sonnenberg et al., 2011b). This suppression against *C. rodentium* infection is mediated by ILCs, which are the main sources of IL-22 in the intestine (Hanash et al., 2012). ILCs have also been associated as a source of IL-22 for defense against DSS-induced colitis and GI graft-versus-host disease (GVHD; Hanash et al., 2012). ILCs have also been

implicated in the pathogenesis of IBD, as they are present within the inflamed intestinal tissue of patients. Studies by Hepworth et al. demonstrate that ILCs regulate $CD4^+$ T cell responses to commensal bacteria within the intestine (Hepworth et al., 2013). Future research is necessary to understand how ILCs manipulate the adaptive immunity to protect against diseases such as IBD. Manipulation of ILCs or the cytokines they produce may provide therapeutic targets in the future for patients with IBD.

IL-22 can also provide pro-inflammatory responses within the intestine. In some cases, such as those involving colonic subepithelial myofibroblasts, increasing IL-22 levels will cause inflammation and hyperproliferation, resulting in negative effects on tissues and the production of pro-inflammatory molecules like IL-1, IL-6, IL-8, IL-11, G-CSF, and GM-CSF (Andoh et al., 2005). Increased IL-22 levels can also recruit pathologic effector cells to the inflamed tissue site, most noticeably in autoimmune diseases in other tissues (Pan et al., 2013).

Host Defense Against Bacterial Pathogens in Intestine

One important mechanism of host defenses in the intestine against bacterial pathogens are the presence of tight junctions that maintain the integrity of the intestinal epithelium, thereby preventing bacterial translocation (Macdonald and Monteleone, 2005), and notably, IL-22 is capable of maintaining these tight junctions (Kim et al., 2012). In addition to the mucin-secreting goblet cells within the intestine, Paneth cells are able to maintain mucosal integrity against pathogens by releasing AMPs, which help contain microorganisms within the GI tract (Takahashi et al., 2001). Zheng and colleagues investigated the role of IL-22 in protecting colonic tissue from infectious colitis with *C. rodentium* (Zheng et al., 2008), paralleling infection by enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) in humans (Mead and Griffin, 1998). EHEC and EPEC both cause infectious diarrhea and carry a high morbidity and mortality, most noticeably in infants and children in developing countries (Zheng et al., 2008). The murine pathogen *C. rodentium* provides an experimental model of infectious colitis in mice. Zheng et al. compared IL-22-deficient mice to wild-type mice infected with *C. rodentium* and found that IL-22-deficient mice had 80–100% mortality during the second week post-infection (Zheng et al., 2008). Wild-type mice in response to infection by *C. rodentium* initially experienced weight loss, but were able to make a full recovery from the infection after ∼6 days (Zheng et al., 2008). Histological analysis of these two groups revealed increased colonic mucosal hyperplasia and increased inflammation in the submucosal tissue in IL-22-deficient mice compared to the wild-type mice (Zheng et al., 2008). Moreover, the site of infection by *C. rodentium* differed in the two groups of mice (Zheng et al., 2008). In the IL-22-deficient mice, bacteria were found deep in the colonic tissue, whereas bacteria in wild-type mice were limited to the superficial layer of the epithelial tissue (Zheng et al., 2008). Furthermore, the authors concluded that IL-22 is the only indispensable cytokine necessary to ensure host defense against *C. rodentium* during the early stages of the infection as opposed to IL-17A, IL-17F, IL-19, IL-20, and IL-24 (Zheng et al., 2008). If IL-22 is absent during initial infection by *C. rodentium*, IL-22 does not provide adequate protection against the bacteria (Zheng et al., 2008). IL-22 knock-out mice infected with *C. rodentium* that were administered IL-22 several days after infection had a high mortality rate compared to infected mice administered IL-22 at the beginning of infection (Zheng et al., 2008). This data is a strong indication that IL-22 is an important cytokine in protecting intestinal epithelial tissue from bacterial pathogens and may provide a therapeutic opportunity against EHEC and EPEC infectious colitis in humans (Zheng et al., 2008).

The microbiota within the intestine contain pathobionts, which are commensal bacteria that can become virulent when homeostasis is disrupted (Hasegawa et al., 2014). For example, when intestinal epithelial tissue is infected with the pathogen *C. difficile*, pathobionts are capable of translocating to other tissues, thereby spreading the infection (Hasegawa et al., 2014). However, IL-22 can control the elimination of enterobacterial pathobionts by facilitating the binding of C3 of the complement pathway to bacteria (Hasegawa et al., 2014). Since little is understood about immune mechanisms in defense against these pathobionts, the discovery of the role IL-22 plays in controlling the pathobionts is important.

IL-22 and Fucosylation in the Intestine

Recently, the IL-22RA1 receptor has also been shown to mediate protection against *C. rodentium* infection in the intestine (Pham et al., 2014). Mice deficient in IL-22RA1 had increased epithelial bacterial translocation of *Enterococcus faecalis* compared to littermate controls. When fucosylated oligosaccharides were administered to the *C. rodentium*-infected IL-22RA1-deficient mice, infection was attenuated and the bacterial diversity of commensals was restored, demonstrating that IL-22RA1 mediates antimicrobial activities and intestinal fucosylation as in **Figure 1** and (Pham et al., 2014). Intestinal epithelial cell fucosylation is catalyzed by fucosyltransferase 2 (Fut2) and is a symbiotic mechanism of host-microbiota interaction, as many bacteria utilize epithelial fucose as a source of dietary carbohydrate (Pacheco et al., 2012; Goto et al., 2014). Pickard et al. demonstrated that Fut2 was expressed by IL-22-stimulated intestinal epithelial cells in the intestine and that fucosylation of the intestinal epithelium occurred in response to Toll-like receptor ligand exposure (Pickard et al., 2014).

Segmented filamentous bacteria (SFB) are an intestinal commensal bacteria in mice that can induce IL-22 expression (Ivanov et al., 2009). Fucosylation in the ileum of SFB-colonized mice has been shown to be dependent on IL-22 and the TNF family member lymphotoxin α (LTα; Goto et al., 2014). IL-22 and LTα are produced by ILC3s to mediate intestinal fucosylation demonstrating that ILC3s may control the intestinal commensals by this mechanism (Goto et al., 2014).

Graft-versus-Host Disease (GVHD)

Graft versus host disease can occur after donor T cells are activated against antigens from the recipient and subsequently attack various organs such as the intestinal tract (Hanash et al., 2012). Multiple investigators have shown that IL-22 derived from the recipient in the liver and GI tract have been shown to reduce mortality and tissue pathology, whereas donor-derived IL-22

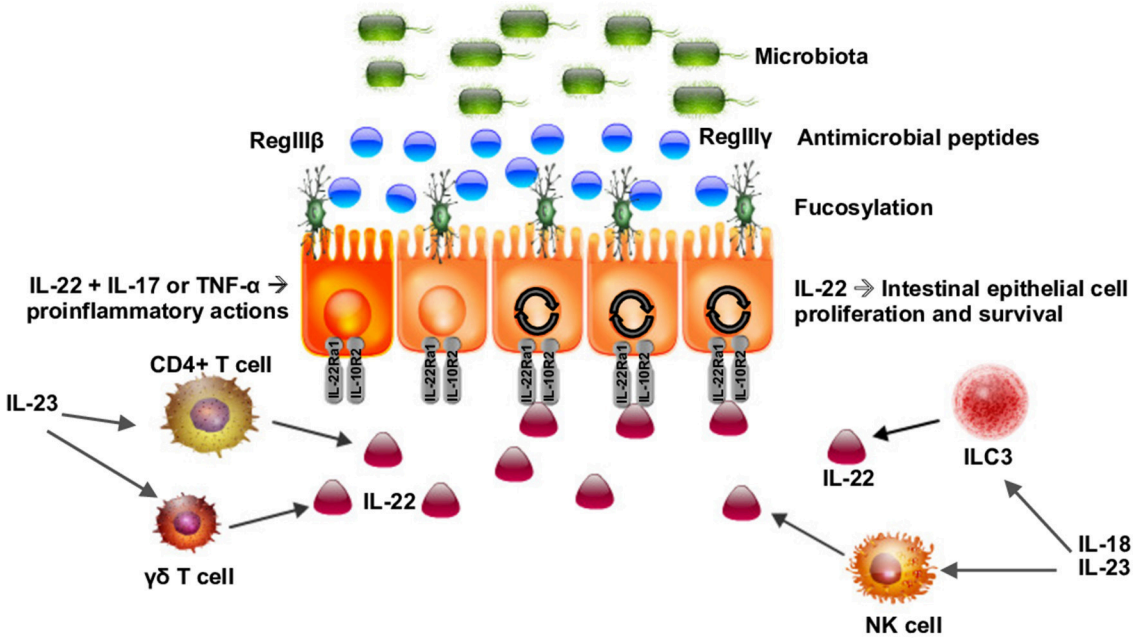

FIGURE 1 | Schematic of IL-22 as a key regulator of the interaction of the microbiota with the intestinal epithelium. IL-22 is produced by CD4+ and γδ T cells as well as type 3 innate lymphoid cells (ILC3s). IL-22 acts on intestinal epithelial cells inducing fucosylation and the release of antimicrobial peptides (AMPs), regulating the microbiota, and maintaining gut barrier homeostasis. IL-22 can act synergistically with IL-17 and TNF-α to activate a proinflammatory response to certain pathogens.

has the opposite effect, increasing mortality and inflammation in target tissues (Hanash et al., 2012; Couturier et al., 2013; Zhao et al., 2013, 2014). Furthermore, recipient-derived IL-22 is produced by ILCs, while donor-derived IL-22 is produced by donor T cells (Hanash et al., 2012). It is thought that the opposing effects may be due to several different mechanisms including the target cells and distinct localizations of donor T cells and recipient ILCs in tissues (Hanash et al., 2012). The potential benefits of IL-22 in GVHD may also be limited since recipient IL-22$^+$ ILCs can be removed by alloreactive donor T cells (Hanash et al., 2012). There is a clinical trial in progress to assess the safety and tolerability of recombinant human IL-22 IgG2-Fc (F-652) in combination with systemic corticosteroids for the treatment of acute gastrointestinal GVHD in hematopoietic stem cell transplantation recipients (ClinicalTrials.gov identifier NCT02406651; Generon Corporation Memorial and Sloan Kettering Cancer, 2016). It has further been noted that there may be a parallel between IL-22 deficiency in GVHD and the condition autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), in which antibodies have the ability to neutralize cytokines such as IL-22 (Kärner et al., 2013; Laakso et al., 2014). As IL-22 levels decrease in patients with APECED, they can develop an increased susceptibility to candida infections (Kärner et al., 2013; Laakso et al., 2014).

IL-22 stimulates the production of molecules from cells within the intestinal epithelium (Peterson and Artis, 2014). For example, molecules like Reg3 and the defensins are thought to be regulated by IL-22 (Kolls et al., 2008; Salzman and Bevins, 2013). However, Paneth cells can produce Reg3 and defensins with limited evidence that IL-22 is solely responsible for this effect (Kolls et al., 2008; Salzman and Bevins, 2013). In addition, a common pathological finding in GVHD is a decrease in the number of Paneth cells (Eriguchi et al., 2012). Therefore, further research is required to understand the role of IL-22 in the regulation and production of these molecules in order to understand pathogenesis of diseases of the GI tract such as GVHD.

Maintenance of the GI Epithelial Barrier

Cross talk between IL-22, ILC3s, and the microbiota within the intestine contributes to the regulation and maintenance of the intestinal epithelial barrier by IL-22 (Sonnenberg et al., 2012). ILC-derived IL-22 has been found to be essential for preventing systemic inflammation, specifically containing species like *Alcaligenes*, a genus of Gram-negative bacteria residing within the mesenteric lymph nodes and Peyer's patches (Sonnenberg et al., 2012). Mice that are deficient in IL-22 display very few epithelial tissue perturbations (Zheng et al., 2008; Sonnenberg et al., 2011b, 2012). However, when these mice are exposed to *C. rodentium*, they develop severe colitis (Zheng et al., 2008; Sonnenberg et al., 2011b). Studies have shown that colitis can be reversed by the introduction of IL-22 (Sonnenberg et al., 2012). In a similar study, mice deficient in ILC3s were found to also be very susceptible to DSS-induced colitis (Sawa et al., 2011). This suggests that ILC3-derived IL-22 plays a protective role in the epithelial tissue against pathogens (Sawa et al., 2011).

The study by Backert et al. (2014) on infectious colitis revealed that the activation of STAT3 in CD4+ cells is necessary for

the expression of IL-22 to facilitate host defense against *C. rodentium* infection. Mice deficient in STAT3 in CD4+ cells mice exhibited no change in the initial course of their infection during the innate lymphoid cell-dependent phase (Backert et al., 2014). However, during the lymphocyte-dependent phase of infection, these mice displayed an augmented distribution of the bacteria as well as significant defects in the intestinal epithelial barrier (Backert et al., 2014). Specifically, the lamina propria was notably significantly deficient in IL-22-producing $CD4^+$ lymphocytes (Backert et al., 2014). This observation suggests that both Th17 and Th22 cells are dependent on STAT3 activation to promote the production of IL-22 (Backert et al., 2014). In mice with active STAT3, the intestinal epithelial barrier was intact and functional, successfully protecting against enteropathogenic bacteria (Backert et al., 2014). These results contribute to our understanding of the mechanisms underlying the role IL-22 in maintenance of the gastrointestinal epithelial integrity.

In bacterial infections caused by *C. rodentium* and *T. gondii*, Munoz and colleagues found that intestinal epithelial cell production of IL-18 is mediated by IL-22 in these two models of intestinal inflammation (Muñoz et al., 2015). IL-18 is required for ILCs to express IL-22, and IL-22 was shown to increase the expression of IL-18 mRNA in the gastrointestinal tissue (Muñoz et al., 2015). In IL-22-deficient mice, there was a reduction in Th1 cells promoting IL-18 expression (Muñoz et al., 2015). Specifically in *C. rodentium* infection, both IL-22 and IL-18 together contribute to barrier defense against the infection (Muñoz et al., 2015). In *T. gondii* infection, IL-18 is required for IL-22 production in the ileum (Muñoz et al., 2015). This study demonstrated the mutual regulation between IL-18 and IL-22 in defense against intestinal infections (Muñoz et al., 2015). These studies contribute to the understanding of the regulation of IL-22 in maintaining gastrointestinal integrity against bacterial pathogens.

In addition to bacterial pathogens, IL-22 can also assist in controlling viral infections. ILC3 production of IL-22 was found to upregulate IFN-λ by intestinal epithelial cells and act synergistically to control rotavirus infection (Hernández et al., 2015), a common diarrheal infection in childhood. The effect of IL-22 on controlling rotavirus replication was dependent on IFN-λR signaling and STAT1 activation and independent of STAT3 (Hernández et al., 2015). These data provide evidence that IL-22 may also provide effective clearance of other GI related viral illness.

The Function of IL-22 in Secondary Lymphoid Tissue

The role that IL-22 plays in secondary lymphoid tissue and mucosa-associated lymphoid tissues (MALTs) including lymph nodes, cryptopatches, isolated lymphoid follicles (ILFs), and Peyer's patches has not been fully elucidated. A study demonstrated that stimulation of Peyer's patches with IL-23 induced NKp46+ NK cells to produce IL-22 (Yoshida et al., 1999; Sun et al., 2000; Eberl and Littman, 2003; Cupedo et al., 2004; Finke, 2005; Tsuji et al., 2008). Furthermore, ILF development is dependent on LTi cells, which produce IL-17, IL-22, and $LT\alpha_1\beta_2$ (Ota et al., 2011). The study by Ota et al. demonstrated that the $LT\alpha_1\beta_2$ pathway has an important role in IL-22 production during infection with *C. rodentium* (Ota et al., 2011). When the $LT\alpha_1\beta_2$ pathway was blocked, colonic IL-22 expression was significantly decreased and exogenous IL-22 administration attenuated colonic damage related to *C. rodentium* infection (Ota et al., 2011). This suggests that IL-22 may be an essential contributor to the maintenance of intestinal lymphoid tissue in the presence of inflammation. Moreover, further studies revealed that the AhR drives IL-22 production, and AhR-deficient mice were not protected against *C. rodentium* infection due in part to a defect in IL-22 production in the lamina propria and Peyer's patches (Zheng et al., 2007). AhR-deficient mice were also found to have a lack of cryptopatches and mature ILFs without affecting formation of Peyer's patches (Zheng et al., 2007). Future studies are warranted to determine whether IL-22 directly or indirectly participates in the maintenance of the GI tract lymphoid tissue and host defense against pathogens in the presence of an infection.

Acute Polymicrobial Sepsis

Weber et al. investigated the role of IL-22 in polymicrobial peritonitis (Weber et al., 2007). The authors infected mice that had been administered recombinant IL-22BP in the form of an Fcγ2a fusion protein (Weber et al., 2007). Mice were treated with IL-22BP-Fc (an IL-22 antagonist; Xie et al., 2000; Dumoutier et al., 2001; Nagalakshmi et al., 2004) 4 hours before polymicrobial septic peritonitis (Weber et al., 2007). Analysis of these mice revealed that there was a significant accumulation of neutrophils and mononuclear phagocytes in conjunction with reduced bacterial load at the direct point of infection (Weber et al., 2007). Additionally, the liver and kidneys of these mice experienced increased bacterial clearance, and kidney injury was ameliorated (Weber et al., 2007). As sepsis progressed in polymicrobial peritonitis, activation of IL-22RA1 and induction of IL-22 produced a pro-inflammatory response that exacerbated the infection (Weber et al., 2007). Bacterial spread and organ failure, which are consequences of polymicrobial peritonitis, appeared to increase due to the role of IL-22 in the progression of sepsis (Weber et al., 2007). They found that both IL-22 and IL-10 were expressed in the presence of sepsis as a consequence of polymicrobial peritonitis (Weber et al., 2007). However, IL-22 was predominantly expressed in the spleen and kidneys, while IL-10 was more widespread in several organs including the spleen, kidney, and liver (Weber et al., 2007). These findings suggest IL-22 influences the production of cytokines and affects the ability of the tissues to provide antibacterial host defense mechanisms in the presence of acute sepsis and peritonitis (Weber et al., 2007). Accordingly, IL-22 has a significant role in acute infection. Furthermore, the IL-22BP derived from Fcγ2a fusion protein can serve as an antagonist to the effects of IL-22 during acute sepsis, and suggests a potential therapeutic intervention for polymicrobial peritonitis (Weber et al., 2007).

CONCLUSIONS

In conclusion, IL-22 plays an important role in the pathogenesis of many intestinal diseases. IL-22 promotes epithelial wound

healing and proliferation of several cell types and in various tissues. Furthermore, the studies described in this review highlight IL-22 as a potential and promising therapeutic target for many gastrointestinal diseases. Extensive research on IL-22 is necessary to fully describe and explain the differences in the ability of this cytokine to provide pro- or anti-inflammatory responses in particular tissues and disease states. A deeper understanding of the regulation and function of IL-22 provides a potential opportunity for the development of novel preventative or therapeutic approaches to many diseases.

FUNDING

DP is supported by R01HL122760. JK is supported by R01HL062052 and R37HL079142. MG is supported by K08DK101608 from the National Institutes of Health and the Children's Hospital of Pittsburgh of the UPMC Health System.

ACKNOWLEDGMENTS

We would like to thank Drs. Jon Piganelli and Elizabeth Novak for critically reviewing the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 01 October 2015
doi: 10.3389/fcell.2015.00062

Mitochondrial dysfunction in inflammatory bowel disease

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Inflammatory Bowel Disease (IBD) represents a group of idiopathic disorders characterized by chronic or recurring inflammation of the gastrointestinal tract. While the exact etiology of disease is unknown, IBD is recognized to be a complex, multifactorial disease that results from an intricate interplay of genetic predisposition, an altered immune response, changes in the intestinal microbiota, and environmental factors. Together, these contribute to a destruction of the intestinal epithelial barrier, increased gut permeability, and an influx of immune cells. Given that most cellular functions as well as maintenance of the epithelial barrier is energy-dependent, it is logical to assume that mitochondrial dysfunction may play a key role in both the onset and recurrence of disease. Indeed several studies have demonstrated evidence of mitochondrial stress and alterations in mitochondrial function within the intestinal epithelium of patients with IBD and mice undergoing experimental colitis. Although the hallmarks of mitochondrial dysfunction, including oxidative stress and impaired ATP production are known to be evident in the intestines of patients with IBD, it is as yet unclear whether these processes occur as a cause of consequence of disease. We provide a current review of mitochondrial function in the setting of intestinal inflammation during IBD.

Keywords: mitochondrial dysfunction, inflammatory bowel disease, intestinal inflammation, metabolic stress, reactive oxygen species, inflammasome, gut-barrier function, autophagy

OPEN ACCESS

Edited by:
Seppo J. Vainio, University of Oulu, Finland

Reviewed by:
Hongmin Qin, Texas A&M University, USA
Pekka Katajisto, Karolinska Institutet, Sweden

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 August 2015*
Accepted: *14 September 2015*
Published: *01 October 2015*

Citation:
Novak EA and Mollen KP (2015) Mitochondrial dysfunction in inflammatory bowel disease. Front. Cell Dev. Biol. 3:62. doi: 10.3389/fcell.2015.00062

Introduction: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a complex, chronic, relapsing, and remitting inflammatory condition of the gastrointestinal tract characterized by symptoms such as diarrhea, bloody stools, abdominal pain, and weight loss (Greco et al., 2011; Indriolo et al., 2011; Rigoli and Caruso, 2014). There are two diseases which fall under the heading of IBD: Crohn's disease and ulcerative colitis. Crohn's disease is characterized by transmural inflammation that may affect any part of the gastrointestinal tract, and presentation of disease is dependent upon both location and severity of inflammation (Podolsky, 2002; Indriolo et al., 2011). Inflammation in ulcerative colitis is limited to the mucosa of the colon and rectum. The pattern of clinical disease in IBD is often cyclical with periods of active inflammation and subsequent remissions (Indriolo et al., 2011). Additionally, there is a strong association between IBD and development of colorectal cancer (Persson et al., 1994; Canavan et al., 2006; Grivennikov, 2013). Although there is no cure for IBD, a range of therapeutics (e.g., corticosteroids, immunomodulators, antibiotics, aminosalicylates, and biologic therapies) is employed to help manage the symptoms of disease. The results of medical treatment are highly variable, and the potential exists for significant morbidity over a long lifetime with disease.

In the United States, it is currently estimated that approximately 1.6 million people suffer from IBD, with as many as 70,000 new cases reported each year (Crohn's and Colitis Foundation of America, 2014). The peak age of onset is between 15 and 35 years with approximately 5–10% of patients diagnosed during childhood (<20 years). IBD is a chronic, lifelong disease that creates a vast financial burden. Previous studies have estimated that the annual direct health care cost for a patient with Crohn's disease is $8265–18,963 and $5066–15,020 per patient diagnosed with ulcerative colitis (Gibson et al., 2008). Extrapolating these figures onto the current prevalence estimates of IBD reveals an estimated annual total direct cost of between 11 and 28 billion dollars (Crohn's and Colitis Foundation of America, 2014).

While the exact pathophysiology of IBD is not yet understood, it is known that the disease is triggered by a complex interaction between genetic, environmental, and immunoregulatory factors. Studies have delineated a clear genetic link to disease. Children of parents affected by IBD have an increased risk of developing the disease (Noble and Arnott, 2008). The risk is significantly higher when both parents have IBD, with disease developing in up to 36% of people with two parents previously diagnosed with IBD (Bennett et al., 1991). Other studies have demonstrated a much higher disease frequency (5–20% increase) amongst first-degree relatives of affected individuals compared to the general population (Russell and Satsangi, 2008). The familial link appears to be stronger in Crohn's disease compared to ulcerative colitis (Tysk et al., 1988; Thompson et al., 1996; Orholm et al., 2000; Halfvarson et al., 2003). Although genetics clearly play a role in disease, the exact nature of genetic predisposition is quite complex, and it is possible that susceptibility to IBD may involve the interaction of several genes. To date, genome-wide association studies (GWAS) have identified more than 160 genetic loci that confer susceptibility to disease (Liu et al., 2015). The fact that genetic polymorphism alone does not predict disease, but merely confers risk of developing IBD highlights the importance that other elements, such as environmental constituents, must also be a contributing factor (Cho and Brant, 2011). Interestingly, the majority of the genetic loci confer susceptibility to both ulcerative colitis and Crohn's disease, calling into question the rigid categorization of IBD subsets.

Although the precise environmental factors that trigger IBD are not known, several risk factors, including antibiotic exposure, stress, dysbiosis, and nonsteroidal anti-inflammatory drug exposure (NSAIDs), are thought to play a role in disease onset and progression (Loftus, 2004; Bernstein et al., 2006; Bernstein, 2008; Molodecky and Kaplan, 2010). Research has demonstrated that IBD is more common in developed countries compared to developing countries, which suggests that many factors associated with the "westernized" lifestyle, such as diet, decreased exposure to sunlight, exposure to pollution and industrial chemicals, may be associated with disease development (Hanauer, 2006). Interestingly, the incidence of IBD in some developing countries (e.g., India and China) is beginning to rise as they become more industrialized (Desai and Gupte, 2005; Zheng et al., 2005). Likewise, migrant studies have revealed that when populations relocate from regions of low IBD prevalence to regions of higher prevalence, they acquire an increased risk of developing disease (Bernstein and Shanahan, 2008; Mikhailov and Furner, 2009). This highlights the importance of environmental factors in the onset and progression of disease in susceptible hosts (Hanauer, 2006).

An evolving body of literature would suggest that predisposing factors converge, resulting in a breakdown of the intestinal barrier and the translocation of luminal antigens. In genetically susceptible individuals, this bacterial translocation triggers a dysfunctional mucosal immune response and promotes inflammation. Although the theory of increased intestinal epithelial permeability as a primary cause of IBD has yet to be proven, it is supported by murine models of experimental colitis (Madsen et al., 1999; Resta-Lenert et al., 2005; Turner, 2009) and some human studies (Söderholm et al., 1999; Zeissig et al., 2007). Since the maintenance of epithelial junction integrity is energy-dependent, it would suggest that mitochondrial function might be central for the appropriate preservation of epithelial barrier function. Interestingly, constituents that have the potential to contribute to IBD susceptibility, such as gastrointestinal infection, and nonsteroidal anti-inflammatory drugs, have also been shown to affect mitochondrial function (Roediger, 1980a; Singh et al., 2009b; Schoultz et al., 2011). Additionally, structurally abnormal mitochondria have been observed in both animal models of intestinal disease (Rodenburg et al., 2008) and in tissues from patients with intestinal inflammation (Nazli et al., 2004). Moreover, processes which influence mitochondrial function, such as autophagy (Travassos et al., 2010), endoplasmic reticulum (ER) stress (Kaser et al., 2008), and the dysregulated production of reactive oxygen species (ROS) (Pavlick et al., 2002; Restivo et al., 2004; Beltrán et al., 2010) have all been implicated in IBD. Despite the present interest in mitochondrial function in the pathophysiology of diabetes (Chowdhury et al., 2013), obesity (Rath and Haller, 2011), and neuromuscular disease (Tarnopolsky and Raha, 2005), little is known about the biological behavior of mitochondria in intestinal inflammation. Here, we summarize the current literature that implicates mitochondrial dysfunction in the pathogenesis of IBD.

Mitochondrial Homeostasis

Mitochondria are membrane-bound organelles that maintain cellular energy production through oxidative phosphorylation (Mitchell and Moyle, 1967). Mitochondria contain a circular genome that encodes 13 proteins and the 22 tRNAs and 2 rRNAs needed to translate those proteins within the mitochondrial matrix. All 13 proteins encoded by mitochondrial DNA (mtDNA) form essential subunits of the respiratory complexes I, III, IV, and V (Anderson et al., 1981; Taanman, 1999). The small mitochondrial genome necessitates that nuclear-encoded genes provide the majority of proteins required for the respiratory apparatus as well as all of the enzymes involved in other cellular biosynthetic and oxidative functions (Anderson et al., 1981; Taanman, 1999). Despite the limited coding-capacity of the mtDNA, mitochondria regulate vital cellular functions aside from energy production, such as the generation of ROS and reactive nitrogen species (RNS), the induction of programmed

cell death, and the transduction of stress and metabolic signals (Galluzzi et al., 2012; Tait and Green, 2012). Thus, it is necessary that mitochondrial dynamics be tightly controlled in order to maintain overall cellular homeostasis.

Mitochondrial biogenesis, the process by which new mitochondria are generated and repaired, plays a significant role in maintaining cellular metabolic homeostasis. Through the growth and division of established mitochondria, the transcription and assembly of new mitochondrial proteins, or the *de novo* synthesis of new mitochondria, mitochondrial biogenesis provides the cell with an adequate pool of healthy mitochondria. This process is influenced by numerous cellular environmental stresses, such as caloric restriction, hypothermia, exercise, cell division, and oxidative stress (Wenz, 2013). Variations in mitochondrial number, size, and mass exist between all cells and are reflective of the current cellular metabolic state (Leary et al., 1998; Leverve and Fontaine, 2001; Pfeiffer et al., 2001; Kunz, 2003). Mitochondrial biogenesis is a complex process, utilizing mitochondrial proteins encoded by both the mitochondrial and nuclear genomes; thus, precise communication between the mitochondria and nucleus is extremely important. Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) is a co-transcriptional regulation factor that is a central modulator of mitochondrial biogenesis (Puigserver et al., 1998). It drives biogenesis by activating various transcription factors, such as nuclear respiratory factor-1 (NRF-1) and nuclear respiratory factor-2 (NRF-2), which not only control the expression of nuclear genes that encode mitochondrial proteins, but also interact with mitochondrial transcription factor A (Tfam) (Jornayvaz and Shulman, 2010), which promotes the transcription and replication of the mitochondrial genome (Virbasius and Scarpulla, 1994).

The competing processes of mitochondrial fusion and fission operate to preserve mitochondrial function or eliminate irreparably damaged mitochondria, respectively. Through their role in regulating mitochondrial dynamics, fusion and fission events fine-tune biological processes central to cell survival, such as ATP generation, calcium homeostasis, and ROS generation. Consequently, they also play a role in apoptosis, mitophagy, cell-cycle progression, and oxygen sensing (Archer, 2013). Highly conserved guanosine triphosphates (GTPases) regulate both processes of fusion and fission (Youle and van der Bliek, 2012; Ishihara et al., 2013). Fusion is regulated by isoforms of two proteins in the outer mitochondrial membrane (OMM), mitofusion-1 and mitofusion-2, and by a dynamin family member, optic atrophy 1 (Opa1) protein, in the inner mitochondrial membrane (IMM) (Youle and van der Bliek, 2012). Mitofusions initiate fusion between neighboring mitochondria through the formation of homodimeric or heterodimeric linkages (Santel and Fuller, 2001; Chen et al., 2003; Hoppins et al., 2007). Opa1 then facilitates the merging of the IMMs (Alexander et al., 2000; Hoppins et al., 2007). Mitofusion-2 also localizes to the ER, where it alters mitochondrial and ER morphology and encourages ER-mitochondria tethering, which enhances calcium signaling (Rojo et al., 2002; de Brito and Scorrano, 2008). Fusion allows for mitochondrial complementation by permitting two mitochondria to fuse and compensate for the defects of each other, thereby generating all of the compulsory machineries for a functional mitochondrial organelle (Archer, 2013). Mitochondria with mtDNA mutations are allowed to fuse with other mitochondria as long as the total mutation burden remains below 80–90% for the cell (Yoneda et al., 1994; Nakada et al., 2001). Mitochondrial fusion is an attempt to buffer brief stresses and fractional defects through the exchange of components in the matrix and intermembrane space (Nunnari et al., 1997; Ono et al., 2001; Chan, 2006; Youle and van der Bliek, 2012).

When mitochondrial damage extends beyond a critical threshold, the quality control mechanisms of fission are initiated. Both ER-mitochondria interactions (Friedman et al., 2011) and the cytosolic protein dynamin-related protein 1 (Drp1) (Chen et al., 2003; Cribbs and Strack, 2009) are conserved features of mitochondrial fission. ER-mitochondria contact points mark the location of mitochondrial division where ER tubules physically wrap around and constrict the mitochondria, presumably to a diameter comparable to the Drp1 helices (Ingerman et al., 2005; Friedman et al., 2011). After ER constriction and upon activation, Drp1 translocates to and multimerizes around the OMM, where it pinches and severs both the IMM and OMM (Legesse-Miller et al., 2003; Lee et al., 2004; Zhu et al., 2004). Fission functions to isolate damaged components of mitochondria by segregating the damaged components of the organelle. After fission, the healthy mitochondrion is able to reincorporate into the network while the damaged mitochondrion is inhibited from reincorporation by a reduction in expression of fusion mediators, such as Opa1. This allows the damaged mitochondria to then be packaged into autophagic vacuoles that are delivered to the lysosome for disposal by the autophagic mechanism of mitophagy (Archer, 2013).

Severely damaged or superfluous mitochondria are degraded by the mitophagy—a specialized form of autophagy that targets individual mitochondria. During mitophagy, whole mitochondria are sequestered into autophagosomes and sent to lysosomes for degradation. Mitophagy is regulated by both the mitochondrial phosphatase and tensin homolog (PTEN)-induced kinase 1 (Pink1) and the cytosolic E3 ubiquitin ligase Parkin (Pellegrino and Haynes, 2015). In healthy mitochondria, expression of Pink1 is repressed by its transport into the IMM and subsequent degradation (Yamano and Youle, 2013; Thomas et al., 2014). However, in damaged mitochondria, Pink1 fails to be imported into the IMM, and instead integrates into the OMM (Geisler et al., 2010; Narendra et al., 2010) with its kinase domain exposed to the cytosol (Zhou et al., 2008), which subsequently recruits Parkin from the cytosol (Geisler et al., 2010; Narendra et al., 2010). Once recruited, Parkin ubiquitinates proteins on the OMM, targeting the mitochondrion for autophagic elimination (Narendra et al., 2008). This mitophagy pathway is also intimately connected with mitochondrial mobility. A major component of mitochondrial transport is mitochondria Rho-GTPase (Miro), a mitochondrial adaptor protein that attaches kinesin motors to the surface of mitochondria. Pink1 and Parkin associate with Miro upon depolarization of the mitochondrial membrane potential, triggering Pink1 to phosphorylate the mitochondrial adaptor protein, subsequently resulting in Parkin-dependent

proteosomal degradation of Miro. Degradation of Miro causes the mitochondrion and the kinesin motor complex to separate, arresting mitochondrial motility (Wang et al., 2011). Arrest of mitochondrial motility, like degradation of mitochondrial fusion proteins, potentially functions to quarantine damaged mitochondria from reincorporating into the mitochondrial network, since static mitochondria are less prone to undergo fusion with other mitochondria (Twig et al., 2010). Homeostasis of the mitochondrial network as well as the proper functionality of the mitochondria is dependent on the cooperation of these cellular functions.

The mitochondrial population must be sustained in order to maintain cellular bioenergetic homeostasis and ensure cellular energy demands are being fulfilled. The plasticity of mitochondrial function and structure is an essential feature to maintaining cellular homeostasis, and indeed, changes in mitochondrial mass have been documented in both health and disease. For example, mitochondrial biogenesis increases in muscle cells upon exercise (Holloszy, 1967). Conversely, research has shown that as mammals age, there is a general decline in both mitochondrial mass and function (Yan and Sohal, 1998; Liu et al., 2002; Chistiakov et al., 2014). There is a wide range of clinical conditions that result from mitochondrial dysfunction, including muscular disorders, cardiomyopathy, diabetes, cancer, deafness, lactic acidosis, and skeletal myopathy (Vafai and Mootha, 2012). In addition, studies show that 1 in every 5000 individuals is affected by a mitochondrial disease (Pfeffer et al., 2012). Mitochondrial dysfunction can affect cell signaling through ROS and metabolites, and can interrupt the intimate physical connections between the mitochondria and other organelles (e.g., ER, etc.). Additionally, mitochondrial dysfunction has severe consequences on the bioenergetics of the cell. Understanding the complex responsibility mitochondria carry in the biology of cell processes and how mitochondrial dysfunction leads to disease can help target specific cellular mechanisms for the treatment and/or prevention of disease.

A variety of conditions and stimuli can alter mitochondrial function. Any disruption of mitochondrial performance can affect overall cellular function and eventually tissue/organ function. Here we review data supporting a role for mitochondrial dysfunction in the development and/or progression of IBD (**Figure 1**). Although a causative role of mitochondrial stress in IBD has not yet been established, the current literature would support a key correlation between mitochondrial function and intestinal inflammation. While we discuss several potential mechanisms by which mitochondrial function may impact disease, it is important to note that all of these processes are interconnected themselves. For example, an alteration in mitochondrial morphology can lead to defective mitochondrial function and communication, build-up of ROS, and activation of the inflammasome, potentially culminating in a disruption of the intestinal barrier, increased permeability, and ultimately intestinal inflammation. Additionally, some of the mechanistic links of mitochondrial dysfunction discussed are more strongly supported by scientific and clinical research (e.g., ROS generation, NLRP3 inflammasome, and autophagy). Nonetheless, it is important to understand how any alteration in the multifaceted functionality of the mitochondrion may contribute to the initiation and propagation of an inflammatory insult.

Mitochondrial Dysfunction in IBD

Form and Function

Mitochondrial form and function are intimately connected in normal cells. The mitochondria are compartmentalized organelles surrounded by two protein-containing phospholipid bilayers. The OMM encloses the entire organelle, and in conjunction with the IMM, separates the IMS and matrix compartments (Perkins and Frey, 2000; Strauss et al., 2008). Both the OMM and IMM contain translocases that function as mitochondrial protein entry ports, directing proteins to the correct subcompartment. The OMM also serves as a central signaling hub for several signal transduction pathways in the cell (Nunnari and Suomalainen, 2012). For example, innate antiviral immunity modulated by mitochondria is dependent upon mitochondrial antiviral signaling (MAVS), an OMM adaptor protein (Seth et al., 2005; Koshiba, 2013); and mitochondria-associated membranes (MAMs) are areas on the OMM where the ER and mitochondrion physically and functionally interact (Pizzo and Pozzan, 2007). The OMM is freely permeable to small molecules, and as such, the intermembrane space contains the same concentration of small molecules (e.g., ions, sugars, etc.) that are also present in the cytosol (Stowe and Camara, 2009). Cytochrome *c*, a protein that is integral to respiration as well as the induction of apoptosis, localizes to the intermembrane space (Koehler et al., 2006; Webb et al., 2006). The IMM, an impermeable membrane that allows for the gated exchanged of metabolites and proteins and undergoes intense folding into cristae to increase the membrane surface area, encloses the matrix compartment. The IMM facilitates lipid trafficking and respiratory complex formation (five complexes in mammals) (Perkins and Frey, 2000; Strauss et al., 2008), which are involved in oxidative phosphorylation and ATP production (Arco and Satrústegui, 2005). The matrix houses numerous copies of the circular mitochondrial genome as well as the machinery needed for its replication, transcription, and subsequent translation of the encoded proteins. Additionally within the matrix is a diverse set of enzymes required for cellular metabolic processes, including fatty-acid synthesis, the Tricarboxylic Acid Cycle (TCA), heme-synthesis, and iron-sulfur cluster formation (Ryan and Hoogenraad, 2007).

There are five primary functions of the mitochondria that are pivotal to mitochondrial form-function dynamics. First, mitochondrial biogenesis regulates the mitochondrial population in order to meet the energy requirements of the cell (Archer, 2013). Second, the cell maintains the health of mitochondria through the process of mitophagy, which eliminates damaged, depolarized mitochondria via lysosomal vacuoles. Mitophagy is facilitated by both the process of fission, which isolates depolarized mitochondria and suppresses fusion mediators, and by inhibiting the reorganization of the damaged mitochondria back into the network (Archer, 2013). Third, mitochondria are mobile organelles that transverse a dynamic network

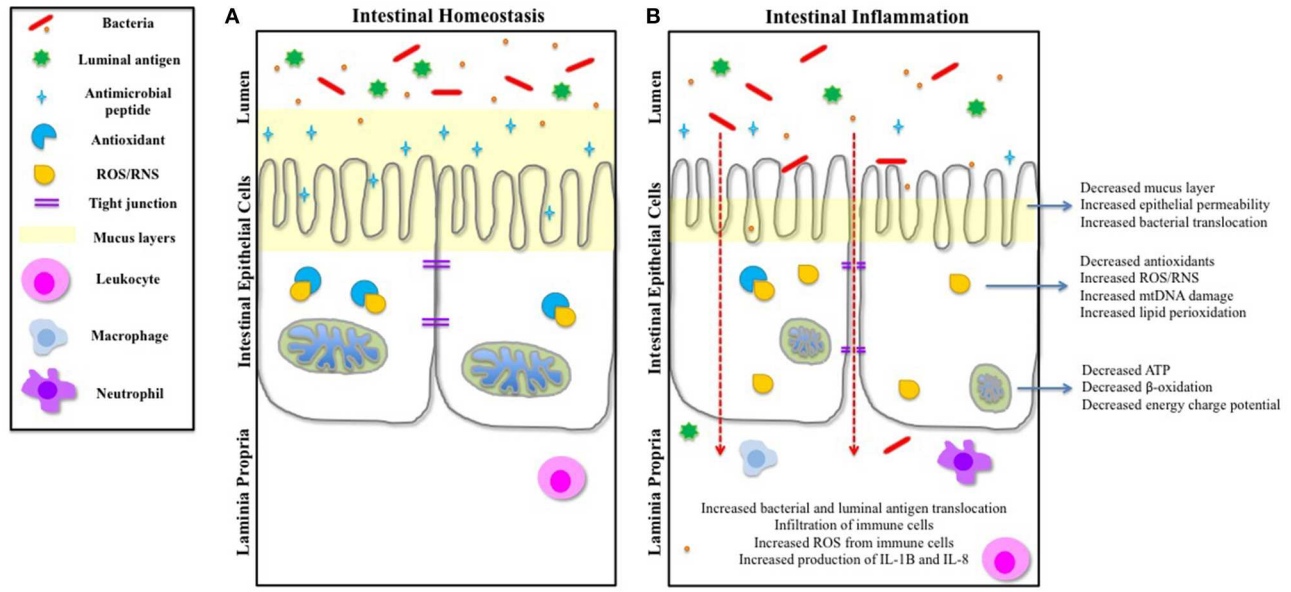

FIGURE 1 | IEC function and intestinal homeostasis can be influenced by mitochondrial dysfunction. (A) During intestinal homeostasis, goblet cells produce a healthy mucus layer that protects the IECs from the contents of the lumen, and Paneth cells produce and release antimicrobial peptides to protect IECs. Mitochondria are dense and contain well-formed cristae. The tight junctions inhibit translocation of luminal antigens across the epithelial barrier. Any basal ROS produced is negated by cellular antioxidants. Leukocytes survey the laminia propria for threats. **(B)** Studies have shown that during the inflammatory conditions of IBD, the mucus layer is reduced and production of antimicrobial peptides is decreased, exposing the intestinal epithelium to the intestinal microbiota and luminal antigens. Mitochondria are swollen and abnormal, and cristae are irregular, resulting in a reduction in ATP production and an increase in ROS. Cellular antioxidants are also decreased, causing a buildup of cellular ROS. There is an increase in epithelial permeability (both transcellular and paracellular) and translocation of bacteria and luminal antigens. This results in an infiltration of immune cells, which also causes an increase of ROS. Both IL-8 and IL-1B are released by immune cells, and immune cell-bacterial interactions further instigates the release of pro-inflammatory mediators, which can feedback onto the IECs and influence other cellular components of the intestinal epithelium.

of dynein and kinesin within the cytosol (Wang et al., 2011). While the relationship between mitochondrial mobility and form and function is not clear, the dynein/dynactin complex is known to regulate the process of fission by recruiting Drp1 to the OMM (Varadi et al., 2004). Fourth, mitochondria are important oxygen-sensing beacons in the cell, and the initial steps in the mechanism of redox signaling depend upon mitochondrial dynamics (Marsboom et al., 2012; Hong et al., 2013). Lastly, mitochondria are linked to the ER through MAMs, enabling these two organelles to communicate through calcium signaling, which has affects on oxidative metabolism and apoptosis (Szabadkai et al., 2004; Denton, 2009; Patergnani et al., 2011). Hence, a minor perturbation in mitochondrial structure or function can lead to mitochondrial dysfunction, which can have deleterious effects on the cell.

Supporting the importance of mitochondrial form and function, enterocytes isolated from patients with IBD have been reported to exhibit swollen mitochondria with irregular cristae (Delpre et al., 1989; Söderholm et al., 2002b; Nazli et al., 2004). Abnormal mitochondrial structure is also seen in intestinal epithelial cells (IECs) from mice subjected to experimental models of colitis (Rodenburg et al., 2008). These morphological changes are suggestive of cellular stress and bioenergetic failure. Indeed, patients with IBD have reduced ATP levels within the intestine (Roediger, 1980a; Kameyama et al., 1984; Schürmann et al., 1999). As would be expected, morphological changes in mitochondria have been shown to result in deficiencies in the β-oxidation of short-chain fatty acids (SCFA) (Halestrap and Dunlop, 1986). It remains unclear, however whether observed changes in mitochondrial structure come as a result of disease or whether they may play a role in the pathogenesis of inflammation.

Intestinal Epithelial Barrier Function

Numerous cellular processes are dependent upon healthy mitochondria for an adequate energy supply. The intestinal mucosa of IBD patients has been demonstrated to be in a state of energy deficiency characterized by low ATP levels and low energy charge potential, (Roediger, 1980a; Kameyama et al., 1984; Söderholm et al., 2002a), calling into question the functionality of this organelle during disease. Indeed, the colonic epithelial cells of patients with ulcerative colitis exhibit mitochondrial alterations before other ultrastructural abnormalities in the epithelium are apparent and before the onset of mucosal inflammation (Delpre et al., 1989; Hsieh et al., 2006). The integrity of the intestinal epithelium, tight junction maintenance, and β-oxidation are key cellular processes within the intestinal epithelium that are not only dependent upon properly functioning mitochondria, but are also known to be altered in animal models of intestinal inflammation and in humans with IBD.

Intestinal Epithelial Cells (IECs)

It is known that IBD is a multifactorial disease, involving the interplay of immune dysregulation, genetic susceptibility, environmental factors, and microbial dysbiosis. The intestinal epithelium comprises the interface between these factors, and thus, may play a vital role in governing this interplay. A key feature of IBD is recurrent damage of the intestinal epithelium concomitant with disruption of the intestinal barrier function (Roda et al., 2010; Henderson et al., 2011; Salim and Söderholm, 2011). The intestinal epithelium is the host's defensive barrier against the luminal microenvironment with discriminatory absorption of nutrients and antigen permeability. The intestinal epithelium is in a constant state of turnover, renewing every 4–5 days and necessitating a considerable supply of energy. The epithelium is comprised of a single layer of different subtypes of IECs, including absorptive enterocytes, mucus-producing goblet cells, enteroendocrine cells, and defensin-producing Paneth cells—all of which differentiate from intestinal epithelial Lgr5+ stem cells (Gibson et al., 1996; Crosnier et al., 2006; van der Flier and Clevers, 2009). The intestinal stem cells are believed to undergo asymmetric division to give rise to transit amplifying (TA) progenitor cells, which are rapidly cycling cells that amplify the progeny of the stem cells, undergoing a limited number of divisions before terminally differentiating into a mature cell lineage and being sloughed off at the villus tip. The cellular structure of the epithelium is organized in space, such that the proliferating stem cells are buried in the crypts and the differentiated mature cells migrate up the surface of the villi (Gibson et al., 1996; Crosnier et al., 2006; van der Flier and Clevers, 2009). Each subset of IECs serves a unique purpose within the epithelium, yet all are critical for intestinal homeostasis and modulating the crosstalk between the microbial community and the circulating immune cells (van der Flier and Clevers, 2009; Noah et al., 2011; Dupaul-Chicoine et al., 2013). Consequently, dysregulation of IEC differentiation has serious effects on the pathogenesis of IBD, and several genes for IEC differentiation have been shown to be perversely expressed in the setting of inflammation (Ahn et al., 2008; Zheng et al., 2011; Coskun et al., 2012). Indeed, depletion of mucus and goblet cells is a characteristic of patients with ulcerative colitis (Jass and Walsh, 2001; Danese and Fiocchi, 2011). Muc2-deficient mice, which lack the gene encoding the major component of mucin, spontaneously develop colitis (Van der Sluis et al., 2006). Likewise, several IBD susceptibility genes are associated with Paneth cell dysfunction. For example, the *Nod2* risk allele for Crohn's disease is associated with a decrease in α-defensin production by Paneth cells in humans (Wehkamp et al., 2004, 2005), and NOD2-deficient mice also exhibit a decrease in α-defensin production (Kobayashi et al., 2005). Paneth cell dysfunction in both humans and mice is also associated with autophagy related 16-like 1 (ATG16L1) (Cadwell et al., 2008, 2009) and X-box binding protein 1 (XBP1) (Kaser et al., 2008), both of which are associated with increased risk of Crohn's disease (Rioux et al., 2007; Kaser et al., 2008). Adolf et al. has shown that by deleting both ATG16L1 and XBP1, mice develop spontaneous CD-like ileitis, which may be a consequence of Paneth cell dysfunction (Adolph et al., 2013). Furthermore, mice lacking caspase-8, a cysteine protease involved in mediating cellular apoptosis, had reduced numbers of goblet cells, no Paneth cells, and also spontaneously developed ileitis (Günther et al., 2011). Thus, defects in intestinal epithelial homeostasis results in an inadequate intestinal barrier defense, which may allow luminal antigens and/or microbes to interact with or violate the intestinal epithelium and consequently cause inflammation (Gersemann et al., 2011). However, the role of mitochondrial dysfunction during IEC differentiation needs to be further evaluated in order to understand the role it may play in the development of intestinal inflammation. Interestingly, Bär et al. demonstrated that altered mitochondrial oxidative phosphorylation activity influences intestinal inflammation in models of experimental colitis using strains of conplastic mice, which have identical nuclear genomes but diverse mitochondrial genomes (Bär et al., 2013). Two strains of conplastic mice, which had increased concentrations of intestinal ATP and augmented oxidative phosphorylation complex activity, were protected from dextran sodium sulfate (DSS)- and trinitrobenzene sulfonate (TNBS)-induced colitis. These mice also had increased proliferation of enterocytes, suggesting that increased intestinal ATP levels (which were due to mtDNA polymorphisms) caused a surge in the turnover rate of the intestinal epithelium—a process that is central to the renewal of the epithelium after exposure to harsh conditions and noxious provocations, such as DSS and TNBS (Bär et al., 2013). This study suggests that increased regeneration of the intestinal epithelium (by means of increased mitochondrial function) is a key factor in combating intestinal inflammation. Indeed, recent clinical evidence has demonstrated that complete mucosal healing is associated with long-term remissions and decreased risk of operative intervention in IBD patients. Mucosal healing also results in the improved mitochondrial structure in the IECs of patients with ulcerative colitis (Fratila and Craciun, 2010). Aside from the providing energy supplies for cell differentiation, there is accumulating evidence that mitochondria play additional roles in cellular differentiation (Maeda and Chida, 2013; Xu et al., 2013; Weinberg et al., 2015). Thus, it is possible that mitochondrial dysfunction could impact IEC differentiation either through energy production or signaling networks, adversely affecting the integrity of the epithelial cell barrier and potentially influencing the development of disease

Tight Junctions

The capacity of the intestinal epithelium to function as a physical, protective barrier is dependent upon tight junctions (TJs), which seal the paracellular space between epithelial cells and polarize the cell membrane. TJs contribute to the integrity of the gut barrier by controlling paracellular permeability and barrier competence of the intestinal epithelium as well as contributing to mucus layer production and infection control (Peterson and Artis, 2014). There are studies that provide a strong link between the development of IBD and altered expression and structural modifications of TJs. Indeed, evidence shows an association between aberrant intestinal permeability and intestinal mucosal inflammation in IBD (Schmitz et al., 1999; Heller et al., 2005; Zeissig et al., 2007). Reports have correlated increased intestinal permeability in first-degree relatives of patients with

IBD, and interestingly, studies have also demonstrated that the spouses of patients with IBD can experience increased gut permeability (May et al., 1993; Söderholm et al., 1999; Breslin et al., 2001; Thjodleifsson et al., 2003). Maintenance of TJ integrity is energy-dependent, and it is not surprising that disruption of the barrier by toxins, pathogens, or noxious stimuli can be initiated by damaged mitochondria (Dickman et al., 2000; He et al., 2000). Certain insults, such as NSAID exposure, are known to disrupt the structure and function of mitochondria, and at least transiently, increase gut permeability (Somasundaram et al., 1997, 2000; Söderholm et al., 1999; Zamora et al., 1999; Basivireddy et al., 2002). Additionally, it has been reported that some patients with Crohn's disease develop immune reactivity against components of their gut microbiome (Pirzer et al., 1991; Duchmann et al., 1996). Consistent with these reports, Nazli et al. demonstrated that treating a cell monolayer with dinitrophenol (an oxidative phosphorylation uncoupler) resulted in cellular internalization of a non-invasive strain of *Escherichia coli*. From this, the authors hypothesized that under metabolic stress resulting from mitochondrial dysfunction, the enteric epithelium loses its ability to distinguish between commensals and pathogens, and as a result, begins internalizing commensal organisms, which can lead to an exacerbated intestinal inflammatory response (Nazli et al., 2004). The mechanism behind developing reactivity to one's own microbiota is not understood, and more research is needed to delineate the role of metabolic stress (e.g., energy deprivation as a result of decreased mitochondrial function) in this process. Studies do suggest that both mitochondrial dysfunction (Lewis and McKay, 2009) and increased gut permeability (De-Souza and Greene, 2005; Deitch et al., 2006) affect the overall competence of the intestinal epithelial barrier, but the stimuli that initiates either process is not known. Nonetheless, these studies lend credence to the implication of epithelial mitochondrial dysfunction as a predisposing factor for an increase in gut epithelial permeability and a loss of gut barrier function, resulting in intestinal inflammation.

β-oxidation

IBD has been suggested to involve a state of energy-deficiency, whereby oxidative metabolism is altered within IECs (Fukushima and Fiocchi, 2004; Saitoh et al., 2008). The SCFA butyrate is the preferred energy source of colonic epithelial cells (Roediger, 1980a; Hamer et al., 2008) and also plays a role in maintaining colonic mucosal health (Hamer et al., 2008). It is a natural nutrient both found in food and produced as an intestinal fermentation by-product of dietary fiber by gut bacteria (Santhanam et al., 2007). Butyrate undergoes catabolic degradation through β-oxidation in the mitochondrial matrix of colonocytes, providing over 70% of the energy demand of the colonic epithelium (Roediger, 1980b). Butyrate metabolism was demonstrated to be impaired in an animal model of colitis (Ahmad et al., 2000), and numerous studies have reported impaired metabolism in the intestinal mucosa of patients with IBD (Roediger, 1980a; Kameyama et al., 1984; Harig et al., 1989; Ramakrishna et al., 1991; Chapman et al., 1994). Similarly, intestinal mucosal inflammation results when butyrate oxidation is inhibited in experimental animals (Roediger and Nance, 1986). Santhanam et al. showed that the mitochondrial acetoacetyl CoA thiolase, which catalyzes the critical last step in butyrate oxidation, was significantly impaired in the colonic mucosa of patients with ulcerative colitis. Furthermore, they conclude that an increase in mitochondrial ROS may trigger this enzymatic defect (Santhanam et al., 2007). Polymorphisms in *SLC22A5*, the gene that encodes for the carnitine transporter OCTN2, is a known risk factor for IBD (Barrett et al., 2008; Singh et al., 2009a). IECs utilize carnitine as a transporter of long-chain fatty acids into the mitochondria for β-oxidation (Rinaldo et al., 2002). Furthermore, genetic ablation of OCTN2 as well as pharmacologic inhibition of intestinal fatty acid β-oxidation results in murine experimental colitis (Roediger and Nance, 1986; Shekhawat et al., 2007). Studies involving the treatment of epithelia cells with dinitrophenol to induce mitochondrial stress resulted in decreased transepithelial resistance and increased bacterial translocation (Lewis et al., 2010)—both of which are features of gut barrier dysfunction. Thus, defective β-oxidation in the mitochondria has deleterious effects beyond energy requirements. Likewise, a dysfunctional gut microbiome or a poor diet may also result in a decrease of butyrate metabolism in the colonic epithelium. Enhanced production of butyrate may potentially benefit the colonic epithelial cells by stimulating an enhancement in cellular homeostasis, including antioxidant and anti-inflammatory roles as well as protective gut-barrier functions.

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Oxidative stress within the intestinal epithelium is thought to play a key role in the pathogenesis of intestinal inflammation (Grisham, 1994; Elson et al., 1995; Conner et al., 1996). Although ROS and RNS are important signaling intermediates involved in a variety of homeostatic molecular pathways (Brown and Griendling, 2009; Gillespie et al., 2009), excessive oxidative stress can provoke cellular damage through the oxidation of proteins, lipids, and DNA, altering their biological functions and potentiating cell death (Andersen, 2004). At baseline, the deleterious effects of ROS generation are negated by a plethora of endogenous antioxidants (Haddad, 2002; Gillespie et al., 2009). The intestinal lumen and epithelium are continuously exposed to noxious stimuli, such as ingested nutrients, local microbes or infections, gastric acid production, and periods of ischemia/reperfusion that have the potential to stimulate the generation oxygen and nitrogen radicals (Parks et al., 1988; Parks, 1989; Young and Woodside, 2001; Sánchez et al., 2002; Biswas et al., 2003; Mazalli and Bragagnolo, 2009). Additionally, the infiltration of leukocytes, monocytes, and neutrophils during inflammation can further enhance intestinal ROS production through both respiratory burst enzymes and prostaglandin and leukotriene metabolism (Babbs, 1992). Several studies have demonstrated increased ROS/RNS levels within the intestinal epithelium of patients with IBD (Kruidenier and Verspaget, 2002; Pravda, 2005; Rezaie et al., 2007) and in murine models of experimental colitis (Girgin et al., 1999; Tham et al., 2002; Narushima et al., 2003; Sundaram et al., 2003; Oz et al., 2005;

Siddiqui et al., 2006; dos Reis et al., 2009; Kajiya et al., 2009; Abdolghaffari et al., 2010; Yao et al., 2010; Lenoir et al., 2011; Ock et al., 2011; Sengül et al., 2011; Borrelli et al., 2013; Arab et al., 2014). High concentrations of oxidized molecules have also been measured in the plasma, serum, exhaled air, and saliva of patients with IBD (Tüzün et al., 2002; Rezaie et al., 2006). Others have shown a positive correlation between oxidative stress and disease severity, suggesting a role in the development and potentiation of inflammation (Rachmilewitz et al., 1995, 1998; Herulf et al., 1998).

In addition to changes in the generation of reactive species, several studies have shown an overall reduction in endogenous antioxidants, such as ascorbate, β-carotene, α-tocopherol, and reduced glutathione, in patients with IBD (Buffinton and Doe, 1995; McKenzie et al., 1996; Schorah, 1998; Sido et al., 1998; Geerling et al., 2000). Interestingly, mice lacking an important antioxidant enzyme, glutathione peroxidase, spontaneously develop symptoms and histologic features similar to those in IBD patients (Esworthy et al., 2001). Moreover, in a murine DSS-induced colitis model, mice subjected to DSS exhibited diminished blood levels of reduced glutathione, which were restored to normal after treatment with various antioxidants (Oz et al., 2005). Levels of catalase, glutathione peroxidase, and superoxide dismutase at baseline have been shown to be lower in the human colonic mucosa, submucosa, and serosa as compared to the human liver (Grisham et al., 1990; Mulder et al., 1991; Buffinton and Doe, 1995) and small intestine (Blau et al., 1999), suggesting a limited capacity to combat oxidative stress in the setting of inflammation. Furthermore, treatment with a mitochondria-targeted antioxidant, MitQ, reduced mitochondrial ROS and protected against experimental colitis in mice subjected to DSS (Dashdorj et al., 2013). Likewise, Wang et al. reported that mitochondrial superoxide was the principal initiator of internalization and transcytosis of a commensal microbes across metabolically stressed epithelium in cell lines and human colonic tissue analyzed *ex vivo*, and that treatment with mitochondrially-targeted antioxidants countered this epithelial barrier defect (Wang et al., 2014). However, it is not yet fully understood if the correlation between ROS/RNS and IBD predicts an actual etiologic relationship for oxidative stress in intestinal inflammation, or if reactive molecular species are merely a consequence of the inflammatory process.

Oxidative stress is thought to exert deleterious effects largely though direct DNA damage and lipid oxidation. Multiple studies have reported increased oxidative DNA damage in the blood and mucosa of patients with IBD (Lih-Brody et al., 1996; D'Incà et al., 2004; Dincer et al., 2007). It has been suggested that these changes may contribute to the increased susceptibility to colorectal cancer that is seen in IBD later in life (Persson et al., 1994; Canavan et al., 2006; Grivennikov, 2013). Colonic mucosal biopsies and plasma from IBD patients also demonstrate an increase in lipid peroxidation products, implying increased ROS production (Pereira et al., 2015). Mice deficient in the antioxidant not only show evidence of increased lipid peroxidation products in both the colon and ileum, but also spontaneously develop colitis (Esworthy et al., 2001), indicating that the ability to combat the oxidative degradation of lipids may be critical in maintaining intestinal homeostasis.

Mitochondria are the most abundant source of ROS in the cell (Beltrán et al., 2010). Under healthy cellular conditions, low levels of ROS are generated and neutralized by the endogenous antioxidant machinery (Haddad, 2002; Gillespie et al., 2009). However, when mitochondria are destabilized by damage or mutations, excessive oxidative stress may result, leading to a reduction of ATP, inhibition of the respiratory chain, and mtDNA damage (Du et al., 1998). Prolonged oxidative stress reduces mitochondrial bioenergetics and homeostasis, promoting cellular damage and ultimately cell death (Scherz-Shouval and Elazar, 2007; Chen and Gibson, 2008). Recent studies have proposed mitochondria as significant cellular drivers and mediators of the inflammatory process (Lee and Hüttemann, 2014). The mitochondria are also a major target of the deleterious effects of oxidative stress, but little is known about how this may lead to inflammation. Understanding the role of mitochondrially-derived ROS in the pathogenesis of IBD may offer key insights into the initiation and propagation of disease.

NLRP3 Inflammasome

Mitochondria are capable of regulating the pro-inflammatory response of the cell through activation of a molecular complex known as the inflammasome. The inflammasome is a multi-protein, caspase-1 activating complex. NLRP3 (NLR family, pyrin domain containing 3) has emerged as critical regulator of intestinal homeostasis (Davis et al., 2014). Formation of the NLRP3 inflammasome is activated by pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) that signify cellular stress, such as extracellular ATP, mtDNA, and ROS (Martinon et al., 2002; López-Armada et al., 2013). Once activated, NLRP3 associates with the adaptor molecule ASC (apoptosis-associated speck-like protein), which contains a caspase recruitment domain (CARD). The associated NLRP3-ASC complex oligomerizes and recruits procapase-1, resulting in formation of the active inflammasome, which in turn causes autocleavage of caspase-1 and release of activated inflammatory cytokines IL-8 and IL-1β (Tschopp, 2011; Zitvogel et al., 2012). Data from murine experimental colitis models and human intestinal specimens reveal that elevated expression of IL-8 and IL-1β is central to the pathogenesis of IBD (Sartor, 1994; Ishiguro, 1999; Monteleone et al., 1999; Kwon et al., 2005; Maeda et al., 2005; Ishihara et al., 2013). IL-8 induces expression of IL-1β and other pro-inflammatory cytokines (Kim et al., 2010), which results in intense intestinal inflammation. IL-1β has been shown to increase gut permeability, which then allows for increased bacterial translocation (Al-Sadi et al., 2012). Furthermore, the secretion of biologically active IL-8 and IL-1β is mediated by caspase-1, which has been reported to play a role in DSS-induced colitis (Siegmund et al., 2001). Bauer et al. demonstrate that DSS induces activation of caspase-1 through NLRP3 inflammasome activation. They further show that NLRP3-deficient mice are protected from DSS-induced colitis, exhibiting a significantly reduced production of pro-inflammatory cytokines as well as improved clinical assessments and histological scores (Bauer

et al., 2010). Additionally, polymorphisms in the *Nlrp3* gene are associated with an increased susceptibility to Crohn's disease (Villani et al., 2009). Schoultz et al. has reported that polymorphisms in the genes encoding both *Nlrp3* and *Card8*, a potent component of the NLRP3 inflammasome, confer increased susceptibility to developing Crohn's disease in Swedish men (Schoultz et al., 2009).

The exact mechanism of NLRP3 inflammasome activation in IBD is not yet known. Several studies have revealed that the NLRP3 inflammasome is involved in murine experimental colitis, and that stimulation of the inflammasome was modulated by mitochondrial ROS (Shimada et al., 2012). However, there remains controversy about the source of ROS that activates intestinal inflammation. Mitochondria are a major, but not the only source of ROS production in the cell. Dashdorj et al. recently published a study implicating mitochondrial ROS as the instigator of inflammation activation (Dashdorj et al., 2013). Consistent with this, other studies have demonstrated that inflammasome activation occurs in mice deficient in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits—a membrane-bound enzymatic complex that functions to generate superoxide, a type of ROS. Additionally, stimulation of the inflammasome also occurs in the mononuclear phagocytes from patients with chronic granulomatous disease, a condition that stems from mutations in the NADPH oxidase subunits (Meissner et al., 2010). Thus, it seems likely that mitochondrial ROS production plays a key role in the intestinal inflammation associated with IBD. However, more research is needed to delineate the extent to which the mitochondria and its concomitant ROS and oxidized mtDNA are involved in the development and progression of intestinal inflammation. Recent studies have begun to appreciate the communication that occurs between mitochondria and pathogen recognition receptors (PRRs) (West et al., 2011). It is interesting to consider the role this communication may play in maintaining immune-microbial homeostasis in the intestinal tract, and how mitochondrial dysfunction may affect the development of intestinal inflammation. Studying the molecular behavior of the inflammasome and its downstream effectors in IBD should add important insights into the mechanistic pathways relevant to the pathogenesis and treatment of disease.

Mitochondrial Communication

Sustaining the mitochondrial population in the cell is central to maintaining cellular bioenergetic homeostasis. Mitochondria have a dedicated repertoire of quality control machinery dedicated to maintaining protein-folding homeostasis. They are composed of four compartments, each of which is a separate protein-folding environment that must be maintained for proper function. Chaperone proteins are localized in the matrix and are required for protein import and promote proper protein folding. In addition to matrix-localized chaperones, there are proteases located in the matrix and IMM, which function to recognize and degrade proteins that fail to fold or assemble properly (Tatsuta and Langer, 2008). Only approximately 10% of the proteins that comprise the electron transport chain are encoded by the mitochondria. The remainder are encoded by the nucleus, translated in the cytosol, and then transported into the mitochondria, where they subsequently assemble into stoichiometric complexes with mitochondrial-encoded proteins (Haynes and Ron, 2010). Thus, it is apparent how cellular stress, such as excessive ROS, mutated proteins, or environmental stress, can negatively affect the protein-folding capacity of the mitochondria, resulting in an accumulation of misfolded proteins or misassembled protein complexes (Ron and Walter, 2007; Ryan and Hoogenraad, 2007). The cell has evolved several quality control pathways to monitor mitochondrial homeostasis and prevent mitochondrial dysfunction. One of these pathways, the mitochondrial unfolded protein response (UPR^{mt}), is a protective response that fosters survival during times of mitochondrial dysfunction or stress by functioning to lessen proteotoxic stress and re-establish protein homeostasis by increasing the population of mitochondrial quality control proteases and chaperones. The UPR^{mt} is a mitochondrial-nuclear cross-talk pathway that, upon communication of unfolded protein stress, activates the transcription factor C/EBP homologous protein (CHOP) (Papa and Germain, 2014), which in turn induces expression of UPR^{mt}-responsive genes (Haynes et al., 2007; Horibe and Hoogenraad, 2007; Baker et al., 2011). It this thought that the UPR^{mt} functions to stabilize and promote the recovery of those mitochondria that are not beyond repair, whereas those organelles that are not salvageable are targeted for mitophagy (Haynes et al., 2013).

Numerous diseases, particularly metabolic and neurogenerative diseases are associated with mitochondrial dysfunction. Some diseases, such as spastic paraplegia, stem directly from mutations that impair mitochondrial function and homeostasis (Casari et al., 1998; Hansen et al., 2002). Most diseases that are associated with mitochondrial dysfunction, though, display characteristics, such as an accrual of mtDNA mutations, augmented ROS generation, and a reduction in ATP output (Haynes and Ron, 2010). All of these features secondarily affect the protein-folding environment of the mitochondria and are common to IBD. However, the exact role UPR^{mt} plays in IBD is just beginning to be uncovered. Rath et al. has recently demonstrated that UPR^{mt} signaling interfaces with the ER unfolded protein response (UPR^{ER}) pathway via double-stranded-RNA-activated protein kinase (PKR). Additionally, IECs were unable to activate *cpn60*, an UPR^{mt} target gene, in PKR-deficient mice subjected to DSS, resulting in resistance to DSS-induced colitis (Rath et al., 2012). This study suggests that the UPR^{mt} has a role in the pathogenesis of IBD, and since it seems that PKR integrates UPR^{mt} signaling into UPR^{ER}, then both mitochondrial and ER protein homeostatic responses might contribute to intestinal inflammation.

The UPR^{mt} is similar to the well-known UPR^{ER}. While both processes contain their own set of chaperones and proteases, and seem to be two distinct pathways, both signaling pathways share the transcription factor CHOP (Horibe and Hoogenraad, 2007) and converge together at PKR (Rath et al., 2012). Moreover, the mitochondria and ER are not only functionally connected, but also physically connected via MAMs, which play a role in calcium homeostasis and lipid biosynthesis. Calcium release at MAMs may advise mitochondria to future apoptotic events

(Szabadkai et al., 2004; Denton, 2009; Patergnani et al., 2011). Accumulation of misfolded proteins in the ER has been suggested to contribute to the development of IBD (Kaser and Blumberg, 2009), and various UPR^{ER} modulators have been correlated to the pathogenesis of IBD (Maloy and Powrie, 2011). Likewise, patients with active IBD normally express augmented ER stress markers in the epithelium of ileum and/or colon (Hu et al., 2007; Shkoda et al., 2007; Heazlewood et al., 2008; Kaser et al., 2008). Furthermore, mice with IEC-specific expression of a dysfunctional UPR^{ER} signaling protein displayed fragmented ER and deteriorated mitochondria (Cao et al., 2014), implying both ER and mitochondrial dysfunction. Both mitochondrial stress and ER stress have been implicated in a set of diseases associated with mitochondrial dysfunction (Fukushima and Fiocchi, 2004; Ozcan et al., 2004; Zhang and Kaufman, 2008; Lim et al., 2009; Haga et al., 2010; Rath and Haller, 2011). It is not known if mitochondrial dysfunction is a result of ER stress and dysfunction; or if a separate, external signal (e.g., diet, microbiome, ROS, etc.) damages the mitochondria, which then consequently, influences the functionality of the ER. It is also remarkable to note that butyrate has been shown to impact mitochondrial pathways and UPR^{ER} signaling in IECs (Fung et al., 2011; Kolar et al., 2011). Since protein homeostasis is sensitive to environmental conditions, it is attractive to speculate that a collaborative UPR (both ER and mitochondria) functions as an innate response to detect harsh changes in the fluctuating intestinal environment. Additionally, given that butyrate is a by-product of the microbial fermentation of SCFAs, it is possible that the composition of the gut microbiota (as well as other luminal antigens) may influence mitochondrial-ER signaling pathways. Nonetheless, it is imperative to consider the contributing role of mitochondria-ER communication in intestinal inflammation.

Mitophagy and Autophagy

Defective autophagy pathways have also been associated with several diseases, including IBD. Cells defective in autophagy accumulate ROS as well as deformed mitochondria (Mizushima and Klionsky, 2007; Saitoh et al., 2008). GWAS have implicated several autophagy genes, including *Atg16l1*, *Lrrk2,* and *Irgm* in the genetic susceptibility to Crohn's disease (Rioux et al., 2007; Barrett et al., 2008; Lees et al., 2011; Umeno et al., 2011). Additionally, previous studies demonstrate that a deficiency in *Atg16l1* results in an increased susceptibility to experimental colitis, abnormal appearance and distribution of Paneth cell granules, and altered mitochondria (Cadwell et al., 2008; Saitoh et al., 2008). Furthermore, Liu et al. showed *Irgm1*-deficient mice exhibited a higher frequency of tubular and swollen mitochondria and increased LC3-positive autophagic vacuoles (Liu et al., 2013). This is consistent with studies that report in humans IRGM localizes to the mitochondria, where it plays a role in mitophagy (Singh et al., 2010). Furthermore, a defect in either ATG16L1 or IRGM has been associated with reduced Paneth cell function, increased susceptibility to bacterial infection, and development of colitis (Cadwell et al., 2008; Saitoh et al., 2008; Liu et al., 2013).

Additionally, prohibitin 1 (PHB), a protein that is important in maintaining normal mitochondrial respiratory function, has been implicated in modulating autophagy. Kathiria et al. demonstrated that PHB regulates autophagy in IECs via intracellular ROS signaling. Moreover, diminished expression of PHB and inhibition of autophagy aggravated mitochondrial depolarization and reduced cell survival, suggesting PHB is an indicator that signals inflammatory stress to the cell, which stimulates autophagy in order to maintain cellular homeostasis and viability (Kathiria et al., 2012). PHB is primarily located on the mitochondria in IECs, and several lines of evidence imply it functions in regulating mitochondrial morphology and function (Artal-Sanz and Tavernarakis, 2009). Interestingly, PHB is decreased in patients with active IBD as well as in animals subjected to experimental colitis (Hsieh et al., 2006; Theiss et al., 2007). Restoration of PHB expression in colonic epithelial cells protected mice from experimental colitis and also exhibited antioxidant properties (Theiss et al., 2009, 2011). Recently, PHB has been shown to interact with the transcription factor STAT3 in colonic epithelial cells and mediate its downstream apoptotic effects. Interestingly, STAT3 has been shown to reside in the mitochondria where it promotes optimal electron transport chain activity, and its activity as a signal transducer has been implicated in IBD (Han et al., 2014).

Mitophagy has also been implicated in the pathogenesis of IBD by a study that revealed an association between single nucleotide polymorphisms in the gene SMAD specific E3 ubiquitin protein ligase 1 (SMURF1) and IBD (Franke et al., 2010). As a regulator of mitophagy, SMURF1 is recruited to damaged mitochondria, where it promotes degradation of the mitochondria by modulating the transport of the autophagic substrate to the autophagosome (Ni et al., 2015). SMURF1 was identified as a crucial mediator of viral autophagy and mitophagy (Orvedahl et al., 2011). However, further studies are needed in order to unravel the part mitophagy plays, beyond normal functions, in IBD pathogenesis. Taken together, these findings suggest autophagy as an important mediator of intestinal homeostasis. Further research is needed in order to delineate the mechanisms of autophagy and their role in intestinal inflammation. Likewise, the interrelation of mitochondrial dysfunction, autophagy, and IBD is still elusive.

Conclusion

Mitochondrial function is undoubtedly crucial to the maintenance of the intestinal epithelium (**Figure 1**). IECs undergo a constant process of cellular turnover and, as such, necessitate a high-energy production at baseline. Aside from supplying the cell with energy, mitochondria also contribute to a plethora of cellular processes, rendering mitochondria central to cell and ultimately organ survival. There are several intestinal inflammatory diseases that involve mitochondrial dysfunction. For example, several clinically significant enteric pathogens that cause intestinal inflammation, including enteropathogenic *E. coli* (EPEC) (Nagai et al., 2005; Kozjak-Pavlovic et al., 2008), *Helicobacter pylori* (Ashktorab et al., 2004; Kozjak-Pavlovic et al., 2008), and *Salmonella typhimurium* (Hernandez et al., 2003; Layton et al., 2005), target effector proteins to the mitochondria of the host cell. Infection with *Citrobacter rodentium* has

been shown to be result in a disruption in mitochondrial function and structure in mice (Ma et al., 2006). Mutations in mtDNA and reductions in cytochrome *c* oxidase activity have also been reported in human colorectal cancer (Heerdt et al., 1990; Alonso et al., 1997; Polyak et al., 1998; Payne et al., 2005; Namslauer and Brzezinski, 2009). IBD, hypothesized to be an energy-deficient disease of the intestinal epithelium, has been demonstrated to be associated with mitochondrial abnormalities of the intestinal epithelium, which occur before the onset of inflammation. Although there is no evidence for a causative association between mitochondrial dysfunction and IBD, here we provide several studies that demonstrate potential links connecting the two. A variety of stimuli and environmental conditions can perturb mitochondrial function, yet the primary stimuli of intestinal mitochondrial stress in IBD have yet to be determined. Since IBD is theorized to require two or more "hits" for the development of disease, it is not illogical to suggest that mitochondrial dysfunction in the intestinal epithelium is an integral component of the intestinal inflammatory process, potentially through effects on epithelial permeability, host-microbiota interactions, or effects on the signaling processes mitochondria are involved in. Nonetheless, the findings reviewed here suggest that the intestinal mitochondria may serve as a novel pharmacological target in the treatment and prevention of IBD, which is consistent with studies published recently using mitochondrial-targeted antioxidants to treat experimental colitis in mice (Dashdorj et al., 2013; Wang et al., 2014). Our understanding of mitochondrial dysfunction in intestinal inflammation is still in its infancy, and there are many more questions than answers. Elucidating the link between mitochondria and IBD will enable the development of new therapeutic strategies aimed at treating the cause of mitochondrial dysfunction, which may potentially prevent and/or treat disease by maintaining both mitochondrial health and homeostasis of the intestinal epithelium.

Acknowledgments

We would like to thank Garret Vincent and Dr. Kellie Cunningham for reviewing the manuscript. We apologize to anyone whose work we left out due to space requirements. This work is supported by a NIH K-award (DK101753) to KPM.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 27 October 2015
doi: 10.3389/fcell.2015.00067

β cell ER stress and the implications for immunogenicity in type 1 diabetes

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Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by hyperglycemia due to progressive immune-mediated destruction of insulin-producing pancreatic islet β cells. Although many elegant studies have identified β cell autoantigens that are targeted by the autoimmune response, the mechanisms by which these autoantigens are generated remain poorly understood. Normal β cell physiology includes a high demand for insulin production and secretion in response to dynamic glucose sensing. This secretory function predisposes β cells to significantly higher levels of endoplasmic reticulum (ER) stress compared to nonsecretory cells. In addition, many environmental triggers associated with T1D onset further augment this inherent ER stress in β cells. ER stress may increase abnormal post-translational modification (PTM) of endogenous β cell proteins. Indeed, in other autoimmune disorders such as celiac disease, systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis, abnormally modified neo-antigens are presented by antigen presenting cells (APCs) in draining lymph nodes. In the context of genetic susceptibility to autoimmunity, presentation of neo-antigens activates auto-reactive T cells and pathology ensues. Therefore, the ER stress induced by normal β cell secretory physiology and environmental triggers may be sufficient to generate neo-antigens for the autoimmune response in T1D. This review summarizes what is currently known about ER stress and protein PTM in target organs of other autoimmune disease models, as well as the data supporting a role for ER stress-induced neo-antigen formation in β cells in T1D.

Keywords: type 1 diabetes, β cell, ER stress, post-translational modification, neo-antigen, autoimmunity

OPEN ACCESS

Edited by:
Seppo J. Vainio,
Oulu University, Finland

Reviewed by:
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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *09 July 2015*
Accepted: *08 October 2015*
Published: *27 October 2015*

Citation:
Marré ML, James EA and Piganelli JD (2015) β cell ER stress and the implications for immunogenicity in type 1 diabetes. Front. Cell Dev. Biol. 3:67. doi: 10.3389/fcell.2015.00067

Abbreviations: APC, antigen presenting cell; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; Ca^{2+}, calcium; CHgA, chromogranin A; CHOP, C/EBP Homologous Protein; EAE, experimental autoimmune encephalomyelitis; eIF2α, α subunit of translation initiation factor 2; ER, endoplasmic reticulum; GAD, glutamic acid decarboxylase; GRP78, 78 kDa glucose-regulated protein; HLA, human leukocyte antigen; IA-2, tyrosine phosphatase-like insulinoma antigen 2; IAPP, islet amyloid polypeptide; ICA69, islet cell autoantigen 69; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; IKKγ, IκB kinase gamma; IP_3R, inositol 1,4,5-trisphosphate receptor; IRE1, inositol-requiring protein 1; JNK, c-jun N-terminal; MAP kinase, mitogen-activated protein kinase; NET, neutrophil extracellular traps; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOD mouse, non-obese diabetic mouse; PAD, peptidylarginine deiminases; PDI, protein disulfide isomerases; PERK, protein kinase RNA (PKR)-like ER kinase; Phogrin, phosphatase homolog of granules from rat insulinomas; PTM, post-translational modification; PTPN22, protein tyrosine phosphatase, non-receptor type 22; ROS, reactive oxygen species; RyR, ryanodine-receptor; SERCA, sacro/endoplasmic reticulum Ca^{2+} ATPase; Tgase2, tissue transglutaminase 2; T1D, type 1 diabetes; UPR, unfolded protein response; XBP-1, X-box binding protein 1; ZnT8, zinc transporter 8.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which insulin-producing pancreatic islet β cells are targeted and destroyed by autoreactive immune cells. Autoimmune recognition of β cells initiates processes that result in loss of β cell mass and the decline of insulin-mediated control of blood glucose levels. Eventually, the remaining β cells become insufficient to maintain normal blood glucose levels, due to reduced β cell numbers and/or to reduced insulin secretion, and chronic hyperglycemia and T1D ensue.

Given the autoimmune mechanisms of β cell destruction, a major underlying risk factor for T1D is a genetic predisposition to autoimmunity. T1D is a polygenic disease, with many genetic loci associated with disease onset. For example, polymorphisms and variants in many genes related to innate and adaptive immune cell function increase susceptibility to autoimmunity, likely by causing failure of central and peripheral immune tolerance mechanisms. With respect to central tolerance, human leukocyte antigen (HLA), which is the genetic variable with the greatest association to T1D onset (Todd et al., 1987; Dorman et al., 1990; Luca et al., 2008), shapes the adaptive immune repertoire by determining which T cells survive thymic maturation and selection. Under normal circumstances, T cells that respond too strongly to self-peptides presented by HLA are deleted or inactivated (Hogquist and Jameson, 2014). In individuals expressing autoimmune-prone polymorphisms within the HLA gene locus, these central tolerance mechanisms fail, permitting autoreactive T cells to mature, and exit the thymus (Fan et al., 2009; Geenen, 2012). With respect to peripheral tolerance, gene variants at other loci such as protein tyrosine phosphatase, non-receptor type 22 (PTPN22) may accelerate T1D onset through mechanisms that have not been fully elucidated (Pociot and McDermott, 2002; Bottini et al., 2004; Luca et al., 2008; Wallis et al., 2009). For example, some studies suggest that, in the context of genetic predisposition to autoimmunity, incomplete antigen presenting cell (APC) maturation may contribute to T1D progression. These immature APCs do not respond normally to growth factors (Serreze et al., 1993) or to inflammatory stimuli (Serreze et al., 1993; Piganelli et al., 1998). As a result, these APCs exhibit defective antigen processing and presentation that activate autoreactive T cells, but do not trigger tolerogenic mechanisms. Such failure in peripheral immune tolerance may exacerbate T1D pathology. However, as stated above, the precise mechanisms by which many of these genetic variants contribute to T1D remain unknown.

Although genetic predisposition is strongly associated with T1D progression, many epidemiological factors suggest that genetic predisposition is not sufficient to drive pathology. First, only a small portion of individuals with HLA predisposition actually progress to T1D (Knip et al., 2005). Second, monozygotic twins demonstrate relatively low concordance for T1D onset (Barnett et al., 1981; Verge et al., 1995). Third, the incidence of T1D is increasing at a rate that cannot be supported by genetic predisposition alone (Onkamo et al., 1999; Gale, 2002; DIAMOND Project Group, 2006). Finally, the age of onset and rate of progression of T1D vary greatly among patients. Together, these data support a role for environmental factors in triggering T1D onset and affecting progression. Among the many environmental triggers associated with T1D onset are viral infection (Atkinson et al., 1994; Horwitz et al., 1998, 2004; Hiemstra et al., 2001; Härkönen et al., 2002; Schulte et al., 2010), β cell exposure to chemicals (Like and Rossini, 1976; Rossini et al., 1977; Takasu et al., 1991a) or reactive oxygen species (ROS) (Piganelli et al., 2002; Tse et al., 2010; Delmastro and Piganelli, 2011; Delmastro-Greenwood et al., 2014), dysglycemia (Sosenko et al., 2009), and inflammation (Mandrup-Poulsen et al., 1987; Held et al., 1990; Jiang and Woda, 1991). Each of the environmental triggers listed here can cause β cell endoplasmic reticulum (ER) stress, suggesting that ER stress may be a common factor in disease onset. However, whether these environmental factors share common pathways to T1D remains unknown.

To understand how these factors lead to the progression of T1D, scientists have studied the non-obese diabetic (NOD) mouse. Mice of this strain develop spontaneous autoimmune diabetes with many similarities to human T1D. These similarities include genetic susceptibility at the HLA locus and other loci, and intra-islet infiltration of immune cells resulting in β cell destruction (Tochino, 1987; Leiter, 1989; Driver et al., 2012). Seminal studies with this mouse model have identified many β cell antigens targeted by the autoimmune response. These murine autoantigens include preproinsulin (Wegmann et al., 1994), glutamic acid decarboxylase (GAD65) (Tisch et al., 1993), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Lieberman et al., 2003), chromogranin A (CHgA) (Stadinski et al., 2010), islet amyloid polypeptide (IAPP) (Delong et al., 2011), zinc transporter 8 (ZnT8) (Nayak et al., 2014), and 78 kDa glucose-regulated protein (GRP78) (Rondas et al., 2015). With the exception of GRP78, these proteins are also confirmed autoantigens in human T1D (Baekkeskov et al., 1990; Keller, 1990; Gorus et al., 1992; Yang et al., 2006; Wenzlau et al., 2007; Gottlieb et al., 2014) along with additional autoantigens found in humans but not yet identified in NOD mice such as tyrosine phosphatase-like insulinoma antigen 2 (IA-2) and IA-2β [also known as phosphatase homolog of granules from rat insulinomas (phogrin)] (Bonifacio et al., 1995; Lan et al., 1996), and islet cell autoantigen 69 (ICA69) (Pietropaolo et al., 1993). However, the precise mechanisms by which these β cell proteins come to be recognized and targeted by the autoimmune response in T1D remain unknown. Recent evidence suggests that some of these proteins undergo post-translational modification (PTM), generating "neo-antigens" with increased immunogenicity (Dunne et al., 2012). But whether such PTMs occur in the β cell, and what cellular processes might give rise to these PTMs in the β cell, remain unknown.

Here, we discuss cellular conditions (both physiological and pathological) that lead to protein PTM. We also review what is currently known about PTM and neo-antigen generation in target organs of other autoimmune disease models. Finally, we review the evidence supporting a role for ER stress-induced PTM in neo-antigen formation in β cells in T1D.

ER STRESS ACTIVATES THE UNFOLDED PROTEIN RESPONSE

The ER is the organelle primarily responsible for folding and PTM of membrane-bound and secreted proteins. To accomplish these tasks, the ER lumen contains the necessary factors to support proper protein folding including molecular chaperones, ATP, an oxidizing environment to support disulfide bond formation, and millimolar concentrations of calcium (Ca^{2+}) (Gething and Sambrook, 1992). Proteins that are properly folded exit the ER and continue toward their intended intra- or extracellular locations. However, improperly folded proteins remain in the ER and, when too many misfolded proteins accumulate, ER homeostasis is disrupted and ER stress ensues. ER stress activates the cytoprotective unfolded protein response (UPR), which acts to relieve ER stress and restore homeostasis by two mechanisms (Hetz, 2012). First, UPR signaling temporarily inhibits the synthesis of new non-chaperone proteins to prevent further burdening the ER machinery. Second, UPR signaling increases the expression of protein chaperones to aid in the folding of the accumulated misfolded proteins in the ER lumen.

During normal ER homeostasis, the chaperone GRP78 [also known as binding immunoglobulin protein (BiP)] binds three protein sensors of ER stress that reside in the ER membrane: protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein 1 (IRE1) (Bertolotti et al., 2000; Shen et al., 2002). Interaction with GRP78 keeps these proteins inactive and thereby inhibits the UPR (**Figure 1A**). However, when misfolded proteins accumulate in the ER, GRP78 releases these protein sensors to bind exposed hydrophobic residues in unfolded proteins. Once free from GRP78, each protein sensor initiates a signaling cascade of the UPR. PERK oligomerizes and becomes activated through autophosphorylation in *trans*. Activated PERK then phosphorylates the α subunit of translation initiation factor 2 (eIF2α) to attenuate mRNA translation and reduce the protein burden in the ER (Harding et al., 2000a,b). ATF6 translocates to the Golgi apparatus where it is cleaved to yield a transcription factor that initiates new chaperone synthesis to aid with folding of accumulated misfolded proteins (Haze et al., 1999). IRE1 oligomerizes and autophosphorylates in *trans*, enabling its endonuclease capability. IRE1 then splices X-box binding protein 1 (XBP-1) mRNA (Yoshida et al., 2001), which encodes a transcription factor that regulates proteins involved in relieving ER stress such as chaperones (Lee et al., 2003) and proteins involved in lipid synthesis to increase ER volume

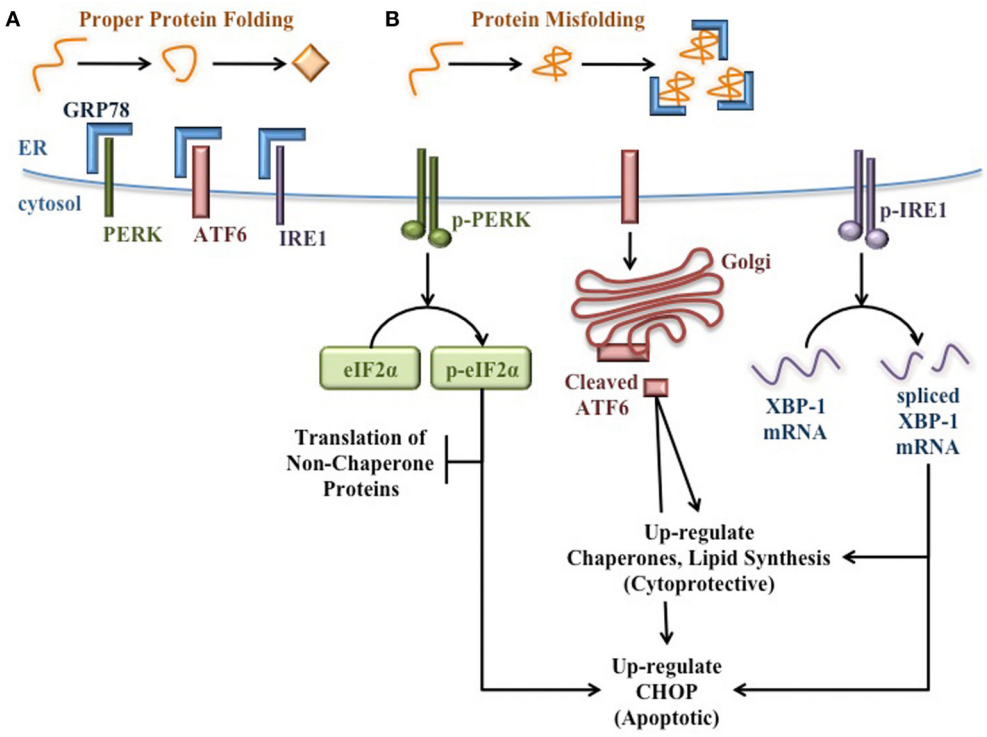

FIGURE 1 | Signaling pathways of the unfolded protein response. (A) When protein folding proceeds normally, the protein sensors of ER stress (PERK, ATF6, and IRE1) are bound and held in their inactive state by GRP78. **(B)** When misfolded proteins accumulate in the ER lumen, GRP78 binds misfolded proteins, thereby releasing the protein sensors of ER stress and allowing for the activation of the cytoprotective UPR. PERK autophosphorylates in *trans*, then activates eIF2α by phosphorylation to attenuate translation of additional non-chaperone proteins. ATF6 translocates to the Golgi apparatus and is cleaved to yield a transcription factor that up-regulates the expression of molecular chaperones to aid in the folding of accumulated proteins in the ER. IRE1 autophosphorylates in *trans* and splices XBP-1 mRNA. The spliced mRNA encodes a transcription factor that up-regulates the expression of additional molecular chaperones and UPR proteins to relieve ER stress. If ER stress is too great or prolonged, the UPR induces expression of pro-apoptotic proteins such as CHOP.

(Sriburi et al., 2004). Through these three signaling cascades, the UPR attempts to reduce ER stress and prevent stress-induced apoptosis (**Figure 1B**).

However, if the burden of unfolded proteins and the subsequent ER dysfunction are too great or too prolonged, these cytoprotective functions of the UPR fail. Under these conditions, pro-apoptotic signaling pathways become activated and ultimately lead to death of the affected cell (**Figure 1B**). For example, the UPR induces C/EBP Homologous Protein (CHOP) expression (Wang et al., 1996), which increases ROS-mediated mitochondrial apoptosis signaling pathways (Zinszner et al., 1998; McCullough et al., 2001).

Thus, ER stress and the UPR have significant effects on cellular function and viability. Even in cells that return to homeostasis following UPR activation, ER stress and dysfunction still have consequences. For example, ER stress often results in the release of Ca^{2+} from the ER lumen to the cytosol. Since high Ca^{2+} concentrations are necessary for protein folding, this efflux of Ca^{2+} negatively affects ER function. Second, ER stress and dysfunction lead to abnormal protein folding and PTM, affecting protein function. Therefore, ER stress, even when temporary, may have important effects on cellular function and physiology.

ER STRESS IS A CONSEQUENCE OF NORMAL β CELL PHYSIOLOGY

All cells undergo some degree of ER stress and activate the UPR in response to improper protein folding or during times of increased protein translation. However, professional secretory cells are uniquely susceptible to ER stress as a result of their normal physiology. In addition to proteins for cellular maintenance, secretory cells are burdened with synthesizing the proteins to be secreted and the proteins required for functional secretory pathways. Thus, the demands of protein translation and folding in the ER of secretory cells are significantly greater than in nonsecretory cells. Although secretory cells contain a more fully developed ER with additional chaperones to compensate for this demand (Shimizu and Hendershot, 2009), the increased ER burden leads to increased occurrence of ER stress.

β cells, like all professional secretory cells, naturally undergo high levels of ER stress as a result of their normal secretory physiology (Araki et al., 2003a; Lipson et al., 2006a,b; Wu and Kaufman, 2006; Fonseca et al., 2007; Ortsäter and Sjöholm, 2007; Eizirik et al., 2008; Volchuk and Ron, 2010; Kim et al., 2012; Teodoro et al., 2012). Indeed, β cells undergo significant ER stress during postprandial glucose-stimulated insulin synthesis (Lipson et al., 2006a,b). β cells increase translation of preproinsulin by 50-fold in response to heightened blood glucose concentrations, reaching a production rate of 1 million molecules of preproinsulin per minute (Scheuner and Kaufman, 2008). These 1 million molecules flood the ER lumen for folding and disulfide bond formation, causing tremendous ER stress. Such cellular processes of dynamic insulin production and heightened ER stress occur from an early age. In XBP-1 splicing reporter mice, the pancreas was the first tissue to exhibit high levels of ER stress and did so as early as 16 days old post birth (Iwawaki et al., 2004). Therefore, normal insulin-secreting physiology alone significantly increases ER stress in β cells.

In addition to the high levels of inherent ER stress, many of the putative environmental triggers associated with T1D may further enhance β cell ER stress. First, Coxsackie viral infection disrupts the ER membrane (van Kuppeveld et al., 1997, 2002, 2005) releasing Ca^{2+} from the ER into the cytosol. Second, β cell exposure to chemicals such as streptozotocin and alloxan cause protein ADP-ribosylation (Sandler and Swenne, 1983) and ROS generation (Heikkila et al., 1976; Takasu et al., 1991b; Bedoya et al., 1996), both of which lead to protein misfolding, and also decrease ER lumen Ca^{2+} concentrations (Kim et al., 1994; Park et al., 1995). Third, β cell exposure to ROS from either extracellular or intracellular sources releases Ca^{2+} from the ER lumen into the cytosol (Favero et al., 1995; Xu et al., 1998; Görlach et al., 2006). Also, dysglycemia leads to increased glucose sensing that, as discussed above, significantly increases insulin production and secretion (Scheuner and Kaufman, 2008). Finally, pancreatic inflammation and cytokine exposure activates c-jun N-terminal (JNK) mitogen-activated protein (MAP) kinase signaling pathways (Wang et al., 2009; Lee et al., 2011). The cellular effects of each environmental trigger exacerbate β cell ER stress. Therefore, although the precise mechanisms by which these environmental triggers accelerate T1D may vary, all the factors listed here can increase β cell ER stress above the normal physiological levels. Therefore, heightened ER stress may be a common factor in early T1D pathogenesis (**Figure 2**).

ER stress and diabetes have been linked in both human and mouse studies. In studies of human islets, ER stress markers

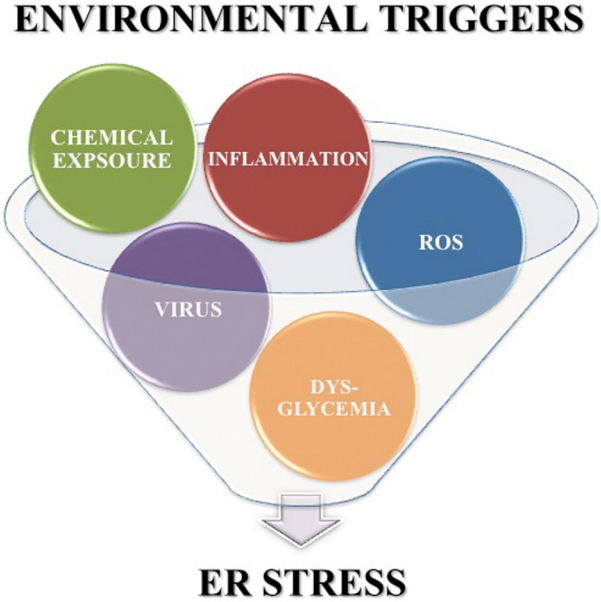

FIGURE 2 | Environmental triggers associated with T1D exacerbate β cell ER stress. Environmental factors such as viral infection, chemicals, ROS, dysglycemia, and pancreatic inflammation are associated with onset of T1D. Each of these environmental triggers of T1D also increases β cell ER stress above the inherently high levels induced by normal β cell physiology.

were increased in islets of T1D patients compared to islets of nondiabetic controls (Marhfour et al., 2012). In the Akita mouse model, the *Ins2*^{C96Y} mutation prevents the formation of a crucial disulfide bond leading to misfolded insulin (Ron, 2002) and high ER stress in these β cells (Ron, 2002; Araki et al., 2003b; Nozaki et al., 2004). This ER stress leads to β cell apoptosis through the activation of CHOP signaling pathways (Oyadomari et al., 2002; Ron, 2002). However, inhibition of CHOP-mediated apoptosis merely delays, but does not halt, β cell loss and disease onset (Oyadomari et al., 2002). These data suggest that apoptosis may not be the only mechanism by which ER stress causes β cell death and diabetes.

ER STRESS ALTERS CA^{2+} CONCENTRATIONS IN THE ER LUMEN AND CYTOSOL

In addition to folding and PTM of proteins, the ER is an important organelle for the maintenance of intracellular Ca^{2+} homeostasis. The ER contains the largest intracellular store of Ca^{2+} and is an important source of Ca^{2+} necessary for regulating a variety of cellular functions both in the ER lumen and in the cytosol (Meldolesi and Pozzan, 1998).

Within the ER lumen, high concentrations of Ca^{2+} are important for proper protein folding. Many molecular chaperones, including GRP78, are Ca^{2+}-dependent (Ma and Hendershot, 2004). In addition, the proteins that facilitate the formation of disulfide bonds [protein disulfide isomerases (PDI)] also require Ca^{2+} (Nigam et al., 1994). To maintain the high concentration Ca^{2+} necessary for ER function, sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) pumps in the ER membrane actively transport Ca^{2+} from the cytosol into the ER lumen (**Figure 3**). These pumps are regulated by existing concentrations of Ca^{2+} in the lumen to prevent ER Ca^{2+} stores from rising too high. Inhibition of these SERCA pumps prevents the movement of Ca^{2+} into the ER, decreasing the function of molecular chaperones and PDI, and increasing the burden of misfolded protein in the ER (Mekahli et al., 2011).

In the cytosol, Ca^{2+} plays important roles in a variety of cellular functions including metabolism, vesicular trafficking, secretion, transcription, and apoptosis (Berridge et al., 2000). Ca^{2+} channels in the ER membrane such as ryanodine-receptor (RyR) and inositol 1,4,5-trisphosphate receptor (IP_3R) release Ca^{2+} from the ER lumen into the cytosol according to its chemical gradient (**Figure 3**). Like the SERCA pumps, the function of these channels is regulated to prevent depletion of the ER Ca^{2+} concentrations (Mekahli et al., 2011).

In spite of the regulation of SERCA pumps and Ca^{2+} channels, the normal Ca^{2+} gradient across the ER membrane is altered during ER stress, leading to decreased Ca^{2+} in the ER and increased Ca^{2+} in the cytosol. These changes in Ca^{2+} concentrations have important effects for the cell. The ER chaperones and PDI necessary for proper protein folding depend on Ca^{2+}, so this imbalance exacerbates ER stress and further activates the UPR. In addition, increased cytosolic Ca^{2+} can cause apoptosis. For instance, Ca^{2+} release from the ER activates the ER-associated procaspase 12 (murine) or procaspase 4 (human), which initiate the caspase cell death pathway (Nakagawa et al., 2000; Hitomi et al., 2004). Also,

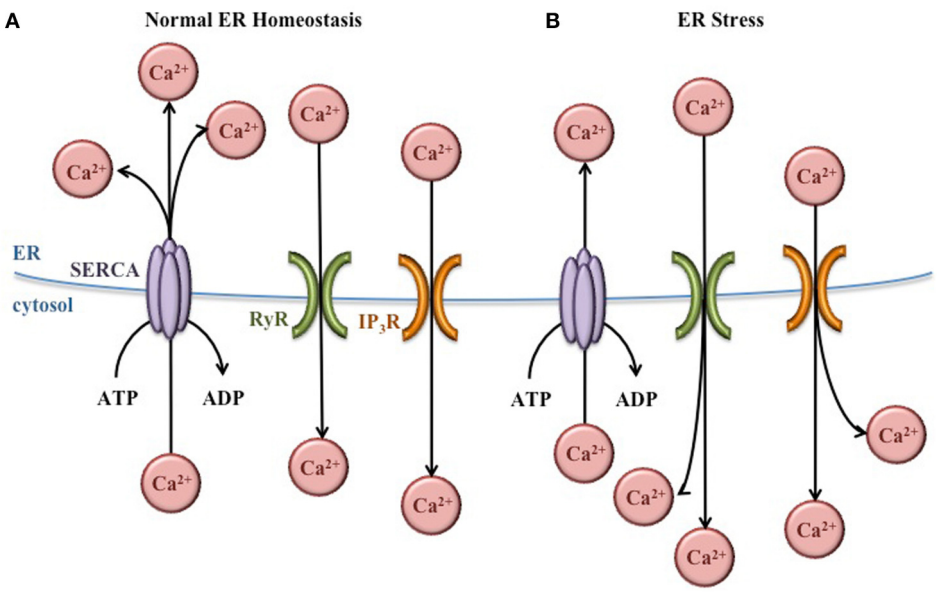

FIGURE 3 | Regulation of ER Ca^{2+} concentrations. (A) Under normal conditions, Ca^{2+} concentrations are higher in the ER lumen than in the cytosol. This balance is maintained by SERCA pumps that bring Ca^{2+} into the ER lumen, and Ca^{2+} channels (RyR and IP_3R) that release Ca^{2+} into the cytoplasm as needed for normal cellular signaling. **(B)** During ER stress, the Ca^{2+} gradient across the ER membrane is disturbed, leading to Ca^{2+} release from the ER and increased Ca^{2+} concentrations in the cytoplasm.

the Ca^{2+}-dependent ER chaperone calreticulin (Michalak et al., 2009) activates caspase 3- and cytochrome c-dependent apoptosis pathways when ER Ca^{2+} concentrations decrease (Nakamura et al., 2000). Furthermore, increased cytosolic Ca^{2+} activates enzymes such as calpain and calciuneurin which activate mitochondria-dependent signaling cascades that ultimately lead to cellular apoptosis (Nakagawa and Yuan, 2000; Gil-Parrado et al., 2002; Kim et al., 2002; Hajnóczky et al., 2003).

Therefore, the maintenance of Ca^{2+} homeostasis is crucial for cellular health and function. Disruption of this Ca^{2+} gradient across the ER membrane has major consequences for ER function and cellular viability.

INCREASED CYTOSOLIC CA^{2+} ACTIVATES POST-TRANSLATIONAL MODIFICATION ENZYMES

While the of activation apoptotic signaling pathways usually requires prolonged ER stress and disrupted Ca^{2+} gradients, other cytosolic Ca^{2+}-depenent enzymes are activated in response to more transient ER stress and heightened cytosolic Ca^{2+} concentrations. In particular, two families of Ca^{2+}-dependent PTM enzymes are activated during ER stress. The activation of these enzymes has important implications for the proteins being folded in the ER.

Tissue Transglutaminase 2

Tissue transglutaminase 2 (Tgase2) is a ubiquitously expressed Ca^{2+}-dependent PTM enzyme that resides in the cytosol (Lesort et al., 1998). Tgase2 becomes activated when Ca^{2+} concentrations in the cytosol rise above normal physiological levels. Indeed, Tgase2 activity requires Ca^{2+} concentrations above what is necessary for normal cellular signaling. As such, Tgase2 usually becomes activated only when cellular homeostasis is disrupted, such as when Ca^{2+} is released from the ER during ER stress (Ientile et al., 2007; Kojima et al., 2010; Wilhelmus et al., 2011; Kuo et al., 2012; Verhaar et al., 2012). Once active, Tgase2 translocates to several intra- and extra-cellular compartments (Park et al., 2010) including the ER (Orru et al., 2003; Wilhelmus et al., 2011; Verhaar et al., 2012) and secretory granules (Russo et al., 2013), to modify proteins by two mechanisms (Facchiano et al., 2006): first, Tgase2 forms ε (γ-glutamyl) isopeptide bonds between gluatmine and lysine residues that crosslink proteins, and second, Tgase2 facilitates the deamidation of glutamine. PTM of proteins by Tgase2 is important for a variety of normal cellular processes (Fesus and Piacentini, 2002; Gundemir et al., 2012). For example, Tgase2 modifies caspase 3 (Yamaguchi and Wang, 2006) and mitochondrial proteins (Fok and Mehta, 2007) to regulate apoptosis, nuclear proteins to regulate gene expression (Ballestar et al., 1996; Lesort et al., 1998; Han and Park, 2000), and extracellular matrix protein to promote cell adhesion (Gaudry et al., 1999; Akimov et al., 2000) and wound healing (Haroon et al., 1999; Stephens et al., 2004; Verderio et al., 2004).

Peptidylarginine Deiminase

Peptidylarginine deiminases (PAD) are another family of Ca^{2+}-dependent PTM enzymes that reside in the cytosol (Vossenaar et al., 2003b). Of the five mammalian isoforms, PAD2 is the most widely expressed, and is the isoform expressed in the pancreas (Takahara et al., 1989). PAD become activated when cytosolic Ca^{2+} concentrations increase to levels 100-fold above normal physiological levels (Takahara et al., 1986; Vossenaar et al., 2003b). When activated, PAD are recruited to various subcellular compartments to modify proteins (Jang et al., 2011). PAD convert arginine to citrulline, which causes a loss of a positive charge in the amino acid sequence (Rogers et al., 1977). This change in charge has significant implications for protein folding, interaction, and function (Tarcsa et al., 1996). PAD play several roles in the context of normal cellular physiology. For example, PAD target IκB kinase gamma (IKKγ) to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation (Lee et al., 2010), target vimentin to regulate cytoskeletal disassembly (Inagaki et al., 1989), and are important in the formation of neutrophil extracellular traps (NET) (Li et al., 2010).

CA^{2+}-DEPENDENT PTM GENERATES NEO-ANTIGENS

Although PTMs are important in normal cellular signaling and physiology, PTM of proteins may contribute to autoimmune disorders. If proteins are modified differently in peripheral tissues than in the thymus, the modified peripheral proteins may act as neo-antigens for which there is no immune tolerance (Doyle and Mamula, 2012). Indeed, a variety of PTMs are implicated in the pathology of several autoimmune diseases (**Table 1**). Importantly, many neo-antigens are formed through PTM by the Ca^{2+}-dependent enzymes Tgase2 and PAD. For example, Tgase2 activity is significantly elevated in celiac disease patients (Bruce et al., 1985). Tgase2 forms intermolecular ε (γ-glutamyl) isopeptide bonds, generating dimers of itself and gliadin as well as oligomers of gliadin (Molberg et al., 1998; Fleckenstein et al., 2004). These complexes are recognized by the immune system as neo-antigens, giving rise to increased T cell responses (Molberg et al., 1998) and anti-Tgase2 antibody production (Dieterich et al., 1997). These immune responses exacerbate the inflammatory conditions in the gut (Halttunen and Mäki, 1999; Barone et al., 2007). Also, in multiple sclerosis, citrullination of myelin basic protein forms a neo-antigen to which T cells respond (Martin et al., 1994). This neo-antigen causes disease in experimental autoimmune encephalomyelitis (the mouse model of multiple sclerosis) (Zhou et al., 1995). Finally, in rheumatoid arthritis, patients develop autoantibodies to the citrullinated forms of many proteins (Schellekens et al., 1998; Masson-Bessière et al., 2001; Vossenaar et al., 2003a, 2004; Burkhardt et al., 2005; Kinloch et al., 2005). These autoantibodies are detected in the synovial fluid of rheumatoid arthritis patients at early stages of disease (van Boekel et al., 2002; Vasishta, 2002), suggesting the importance of these PAD-generated neo-antigens for disease progression.

Although Tgase2- and PAD-mediated PTMs are known to generate neo-antigens, little research has been conducted regarding the precise mechanisms by which these pathological

TABLE 1 | Neo-antigens formed by PTM in autoimmune diseases.

Disease	Autoantigen	PTM	References
Celiac disease	Gliadin	Deamidation	Molberg et al., 1998
Collagen-induced arthritis	Type II collagen	Glycosylation	Corthay et al., 1998
		Hydroxylation	Corthay et al., 1998
Multiple Sclerosis/EAE	Myelin basic protein	Acetylation	Zamvil et al., 1986
		Citrullination	Martin et al., 1994
	Myelin oligodendrocyte glycoprotein	Malondialdehyde	Wållberg et al., 2007
	αB-crystallin	Phosphorylation	van Stipdonk et al., 1998
Rheumatoid Arthritis	Filaggrin	Citrullination	Schellekens et al., 1998
	Fibrin	Citrullination	Masson-Bessière et al., 2001
	Fibrinogen	Citrullination	Vossenaar et al., 2003a
	Vimentin	Citrullination	Vossenaar et al., 2004
	Collagen	Citrullination	Burkhardt et al., 2005
	α-Enolase	Citrullination	Kinloch et al., 2005
Systemic lupus erythematosus	Small nuclear ribonucleoprotein particle	Isoaspartylation	Mamula et al., 1999
	70 kd subunit of U1 small nuclear ribonucleoprotein particle	Phosphorylation	Monneaux et al., 2003
	Lupus La protein	Phosphorylation	Coudevylle et al., 2006
	SmD1/SmD3	Methylation	Brahms et al., 2000

PTMs arise in the particular cells and tissues targeted in these autoimmune disease models. However, Tgase2 and PAD, as described above, become activated under conditions that cause significantly elevated cytosolic Ca^{2+}. The main cause of significantly elevated Ca^{2+} is cellular stress, especially ER stress. Therefore, ER stress may give rise to neo-antigen formation through abnormal Ca^{2+}-dependent PTM of endogenous proteins.

T1D AUTOANTIGENS EXHIBIT INCREASED IMMUNOGENICITY AFTER PTM

Although it is well established that PTM of endogenous proteins forms neo-antigens that initiate and exacerbate the autoimmune response in many autoimmune diseases (**Table 1**), the role of PTM in β cell autoantigen formation long remained unexplored. However, in the last 10 years, many seminal studies have demonstrated that known murine and human β cell autoantigens exhibit greater immunogenicity after PTM (**Table 2**). For example, T cells from a human T1D patient recognized an oxidized epitope of proinsulin (Mannering et al., 2005). These T cell responses depended on the formation of a vicinal disulfide bond, as replacement of either cysteine with a serine residue abolished T cell responses against this peptide. In addition, PTM by the Ca^{2+}-dependent enzymes Tgase2 and PAD increases the immunogenicity of several β cell proteins.

TABLE 2 | Neo-antigens formed by PTM in T1D.

Autoantigen	PTM	References
Proinsulin	Oxidation	Mannering et al., 2005
CHgA (WE14)	Crosslinking/ Isospeptide Bond	Delong et al., 2012; Gottlieb et al., 2014
Preproinsulin	Deamidation	van Lummel et al., 2014
ICA69	Deamidation	van Lummel et al., 2014
ZnT8	Deamidation	van Lummel et al., 2014
Phogrin	Deamidation	van Lummel et al., 2014
IA-2	Deamidation	van Lummel et al., 2014
IGRP	Deamidation	van Lummel et al., 2014
GAD65	Citrullination	McGinty et al., 2014
	Deamidation	McGinty et al., 2014; van Lummel et al., 2014
GRP78	Citrullination	Rondas et al., 2015

Chromogranin A

The first β cell autoantigen shown to elicit greater immune responses after Ca^{2+}-dependent modification was the WE14 peptide of chromogranin A (CHgA) (Delong et al., 2012). The authors had previously demonstrated that the BDC2.5 diabetogenic $CD4^+$ T cell clone recognizes WE14 (Stadinski et al., 2010). However, exceptionally high peptide concentrations were required for full T cell activation. In this study, Delong et al. demonstrated that treatment of WE14 with Tgase2 generated a covalently cross-linked peptide that is preferentially presented to BDC2.5 T cells, thereby increasing proliferation and IFNγ production. In addition, splenocytes isolated from pre-diabetic NOD mice responded more strongly to Tgase2-modified WE14 than to the native peptide. A subsequent study showed that WE14 was recognized by T cells from human T1D patients, and that treatment of WE14 with Tgase2 increased the response elicited from these T cells (Gottlieb et al., 2014). This confirmed the relevance of this modified β cell antigen to human T1D. Together, these studies demonstrated that Tgase2-modification of CHgA contributes to the strong activation of autoreactive immune cells in T1D.

Preproinsulin

The deamidation of glutamine by Tgase2 also modulates the recognition of β cell antigens. In a recent study, deamidated peptides from many islet proteins were eluted from T1D-associated HLA-DQ proteins (van Lummel et al., 2014). These Tgase2-modified peptides bound more strongly than their unmodified counterparts to HLA-DQ molecules. Among these, a Tgase2-modified peptide from preproinsulin elicited responses

from $CD4^+$ T cells from a new-onset T1D patient. This study therefore identified novel islet peptides that become neo-antigens through PTM. This study also demonstrated stronger binding to disease-associated HLA molecules as one mechanism by which β cell neo-antigens elicit stronger autoimmune responses.

GAD65

Another β cell protein shown to elicit greater immune responses after PTM is GAD65. Modification of multiple GAD65 peptides by either Tgase2 (deamidation) or PAD (deimidation) increased immunogenicity (McGinty et al., 2014). These peptides bind MHC class II molecules more strongly than the native peptides. Furthermore, T cells that recognize the modified peptides were present at higher frequencies in human T1D patients than in HLA-matched control subjects. These T cells responded to Tgase2-modified peptides of GAD65 more strongly than to the unmodified peptides and displayed a disease-relevant memory phenotype. These data demonstrated a role for Ca^{2+}-dependent PTM in increasing immunogenicity of GAD65 peptides, and further supported a role for PTM-mediated neo-antigen generation in human T1D.

GRP78

Most recently, citrullinated GRP78 was identified as an autoantigen in diabetic NOD mice (Rondas et al., 2015). $CD4^+$ T cells from diabetic NOD mice secreted significantly higher IFNγ in response to citrullinated GRP78 compared to T cells from non-diabetic mice. In addition, new-onset diabetic NOD mice exhibited higher titers of autoantibodies that recognize modified GRP78 compared to age-matched non-diabetic mice. Importantly, these T cell responses and α-GRP78 autoantibodies specifically recognized the ctirullinated peptide, not the native peptide, demonstrating the relevance of PTM to the generation of this neo-antigen. This study, therefore, identified modified GRP78 as a novel autoantigen in the NOD mouse model of T1D.

Together, these studies demonstrate that, as in other autoimmune disorders, PTM enhances the immunogenicity of several known autoantigens in T1D. However, these studies were conducted with synthetic peptides that were modified *in vitro* or designed to mimic modified sequences. Whether the β cell proteins from which these peptides are derived undergo PTM within the β cell remains unknown. In addition, the mechanisms by which Tgase2 and PAD might be activated in the β cell remain undefined. However, as we have discussed here, Tgase2 and PAD are both Ca^{2+}-dependent and known to be activated during ER stress. β cells inherently undergo particularly high levels of ER stress, which may be further increased upon exposure to environmental triggers of T1D. This high ER stress may activate Tgase2 and PAD to modify endogenous β cell proteins, generating neo-antigens. Therefore, β cell autoantigens may become immunogenic due to ER stress-induced PTM.

CONCLUSION

Many elegant and seminal studies have demonstrated that peptides derived from β cell autoantigens become more immunogenic after PTM (Mannering et al., 2005; Delong et al., 2012; Dunne et al., 2012; Gottlieb et al., 2014; McGinty et al.,

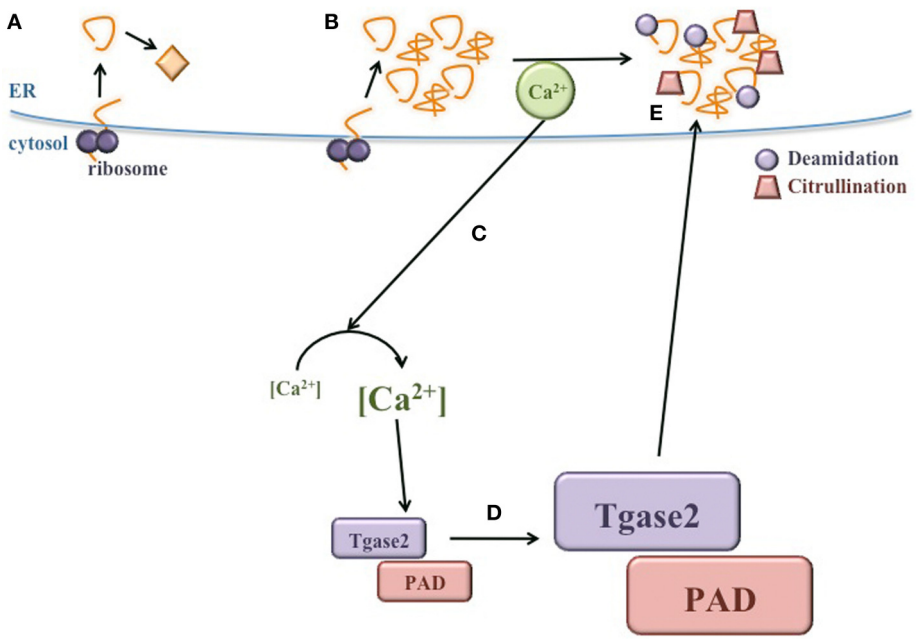

FIGURE 4 | ER stress induces neo-antigen formation in β cells. (A) During normal conditions, proteins are translated and properly folded in the ER lumen. **(B)** During ER stress, proper protein folding is inhibited and misfolded proteins accumulate. **(C)** Ca^{2+} is released from the ER, significantly increasing the concentration of cytosolic Ca^{2+}. **(D)** Heightened Ca^{2+} in the cytosol increases the activity of PTM enzymes, such as Tgase2 and PAD. **(E)** Activated Tgase2 and PAD modify β cell proteins, generating neo-antigens for the autoimmune response in T1D.

2014; van Lummel et al., 2014; Rondas et al., 2015). However, the mechanisms by which these neo-antigens are modified in the β cell have not yet been elucidated. Here, we propose that the normal physiology of the β cell, together with the exposure of β cells to a variety of environmental factors, significantly raises ER stress, leading to the release of Ca^{2+} from the ER lumen into the cytosol. This Ca^{2+} flux may activate cytosolic PTM enzymes, which could modify β cell proteins, generating neo-antigens (**Figure 4**). Because islet β cells are inherently susceptible to high ER stress, these PTMs may occur in all β cells in all individuals. Therefore, T1D onset may not be determined by whether these neo-antigens are generated, but perhaps by genetic predisposition to autoimmunity. Individuals without a genetic predisposition to autoimmunity do not experience a failure of immune tolerance due to central and peripheral mechanisms that maintain immunological tolerance. Thus, the presentation of ER stress-induced modified neo-antigens by APCs may not activate peripheral T cells and T1D may not occur. In contrast, individuals that do harbor genetic predispositions to autoimmunity experience defects in mechanisms of immune tolerance. In these individuals, presentation of modified neo-antigens by APCs could activate autoreactive T cells and cause autoimmune destruction of β cells.

Once the autoimmune response is initiated, the effects of β cell ER stress are magnified. ER stress progressively increases with immune infiltration into the islet (Tersey et al., 2012). Heightened ER stress could lead to increased cytosolic Ca^{2+} and increased activity of Tgase2 and PAD. Recent studies have shown that Tgase2- and PAD-mediated PTMs increase the immunogenicity of peptides derived from known β cell autoantigens (**Table 2**). Therefore, as β cell ER stress progressively increases, these ever-more active enzymes may modify proteins beyond their physiological targets, including known β cell autoantigens. These neo-antigens could be processed and presented by APCs to T cells in draining lymph nodes. Activated immune cells returning to the islet may further increase β cell ER tress by two mechanisms. First, activated immune cells secrete cytokines that directly increase ER stress. Second, immune-mediated destruction reduces β cell mass, requiring the remaining β cells to produce more insulin per cell and augmenting the ER stress in each β cell. Increased ER stress likely leads to the generation of additional neo-antigens, further fueling the autoimmune response. Therefore, once pathology is initiated in T1D, the cycle of ER stress and neo-antigen generation likely hastens the onset of T1D and continues until the β cell mass is fully destroyed.

The recent studies that have identified modified β cell peptides as neo-antigens have opened important new areas of research in the field of T1D. Additional studies to confirm the cause of increased ER stress in the β cell, and to establish the role of ER stress in the generation of these neo-antigens, will further advance the field. In particular, understanding how these neo-antigens arise in β cells will identify opportunities for therapeutic intervention before the β cell mass is destroyed. For example, therapies that aid in proper protein folding or otherwise reduce ER stress may prevent the formation of neo-antigens. Alternatively, therapeutic agents that promote the degradation of abnormal proteins may remove neo-antigens from β cells. Either therapeutic mechanism may prevent immune-mediated recognition of β cells. Indeed, therapeutic agents that reduce ER stress or degrade misfolded proteins are effective in other disease models (Boyce et al., 2005; Ozcan et al., 2006; Harris and Rubinsztein, 2011; Zode et al., 2011; Bachar-Wikstrom et al., 2012; Hasnain et al., 2012; Engin et al., 2013; Jiang et al., 2015). Similar treatments in T1D models may reduce ER stress-induced neo-antigen formation in β cells, preventing immune destruction of β cells and preventing onset of T1D.

AUTHOR CONTRIBUTIONS

MM, EJ, and JP contributed to the composition of this manuscript.

FUNDING

This work was supported in part by the Juvenile Diabetes Research Foundation (3-PDF-2014-213-A-N to MM, 2-SRA-2014-297-Q-R to EJ, and 2-SRA-2014-296-Q-R to JP), the American Diabetes Association (1-12-BS-161 to JP), and Children's Hospital of Pittsburgh of the UPMC Health System (Cochrane-Weber Endowed Fund to JP).

ACKNOWLEDGMENTS

The authors thank Gina M. Coudriet, Ph.D., and Dana M. Previte (both of University of Pittsburgh) for review of this manuscript.

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