Diabetic Complications Consortium

Application Title: Nephron progenitor culture technology for ex vivo nephrogenesis

Principal Investigator: Leif Oxburgh

1. Project Accomplishments:

In work supported by NIH and DoD funding we have developed culture conditions with which primary embryonic nephron progenitor cells (NPCs) can be expanded in the undifferentiated state. Using this technology it is possible to generate cell numbers in the hundreds of millions to billions required for bioengineering applications. However, the cost of culture has been a major limitation. The goal of this Diacomp Pilot project was to systematically test medium formulations and substrates with the goal of reducing costs and making this culture system more accessible for large-scale cell production. Using a systematic approach we have reduced the cost to approximately half and we have collected important data on which extracellular matrices (ECM) will allow cells to proliferate in the undifferentiated state.

2. Specific Aims:

A schematic representation of the approach to the study is shown in Figure 1.

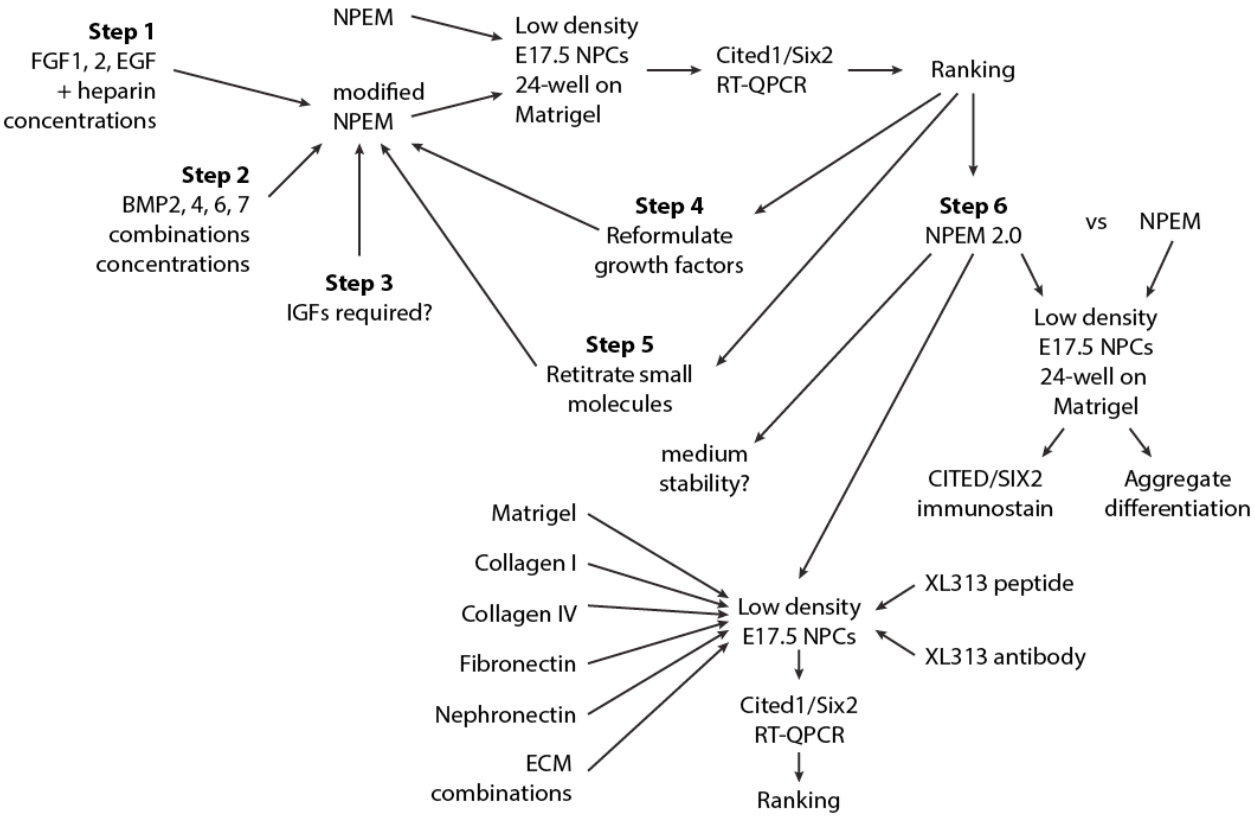

Figure 1: Flow chart for systematic analysis of nephron progenitor culture conditions.

Aim 1: Define a culture medium formulation that maximizes proliferation of undifferentiated nephron progenitor cells.

Results: Key components FGF and BMP were titrated and cheaper substitutes were evaluated (Fig. 2AB). Based on this and retitration of the cheaper components we established a formulation for Nephron Progenitor Expansion Medium (NPEM) 2. Comparison of the original NPEM (NPEM 1) versus the new formulation (NPEM 2) reveals comparable levels of undifferentiated NPC markers Cited1 and Six2 (Fig. 2C). Expression of the differentiation marker LEF1 was slightly elevated in NPEM 2. Cell numbers were comparable as was RNA yield (Fig. 2D). Immunostaining for CITED1, SIX2, and LEF1 revealed comparable expression between NPEM 1 and 2. To test the potential of propagated cells to differentiate, they were expanded to passage 3, then aggregated and exposed to high concentrations of CHIR, which activates β-catenin-mediated WNT signaling. Histological comparison reveals vigorous tubule differentiation and expression of the differentiation marker LTL (Fig. 2F). Medium could also be frozen and stored for up to a month and remain functional (Fig. 2G).

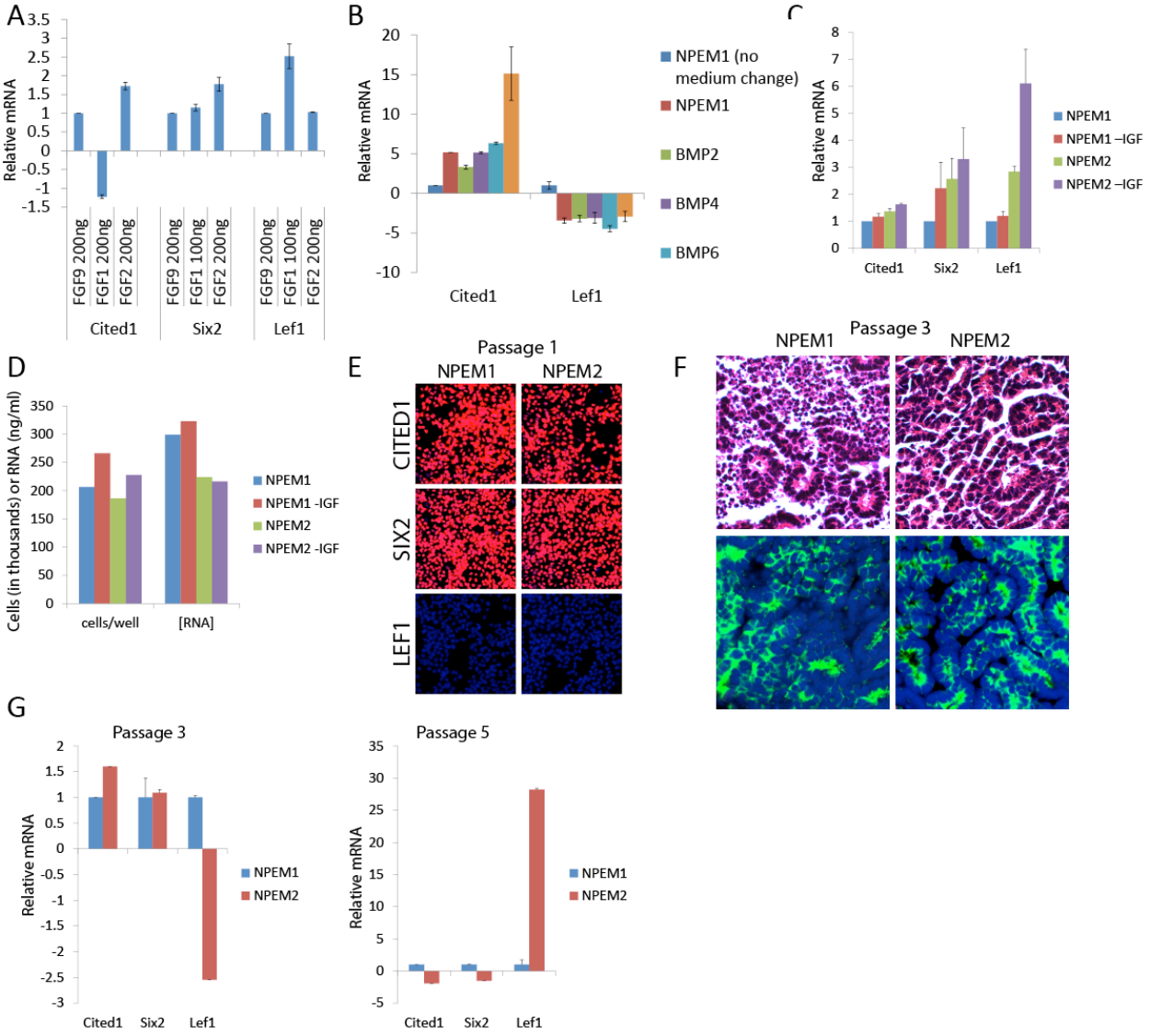

Figure 2: The effects of altering NPEM components on expansion and differentiation of nephron progenitors. (A) qPCR of progenitor markers from optimal concentrations of FGFs determined

by titration after 4 days in culture. FGF20 not shown due to lack of cellular expansion at all concentrations tested. (B) Testing of BMP ligands (50ng/ml) on progenitor marker expression by qPCR after 4 days in culture. Data are shown relative to NPEM1 with no medium change to compare the stability of BMP in NPEM1 with media changes. (C) Comparison of NPEM1 and NPEM2 for progenitor marker expression by qPCR after 4 days in culture. The presence or absence of IGF1/2 was additionally tested. (D) Comparison of NPEM1 and NPEM2 cell number after 4 days in culture. The amount of RNA correlates approximately with cell number. (E) Immunostaining for progenitor markers after 4 days in culture. (F) H&E (top) and E-cadherin immunostaining (bottom) of 3D aggregates of progenitors treated with CHIR that were initially grown in monolayer for 4 growth periods (3 passages). (G) qPCR for progenitor markers after passages 3 and 5. Medium used was frozen for 1 month prior to assay and indistinguishable from unfrozen (not shown). Table 1 shows the cost comparison, which reveals an approximately 50% reduction in cost for NPEM 2.

Table 1: Cost per 100ml nephron progenitor medium

	NPEM1	NPEM1 (bulk order)
APEL medium	129	129
FGF9	204	204
BMP4/7	256.67	71.37
Y27632	66.9	66.9
LDN193189	0.4	0.4
CHIR99021	5.57	5.57
IGF1	1.99	1.99
IGF2	0.72	0.72
Total cost:	665.25	**479.95**

	NPEM2	NPEM2 (bulk order)
APEL medium	129	129
FGF2	149	29.55
BMP7	225	21.29
Y27632	66.9	66.9
LDN193189	0.4	0.4
CHIR99021	5.57	5.57
IGF1	1.99	1.99
IGF2	0.72	0.72
Total cost:	578.58	**255.42**

Aim 2: Develop an extracellular matrix substrate for nephron progenitor cell culture that promotes proliferation of undifferentiated cells.

Results: Matrigel has been the substrate of choice to maintain NPCs in the undifferentiated state. Early work on NPC culture that preceded development of NPEM led us to believe that fibronectin (FN1) was the most realistic candidate for a pure ECM molecule that could support undifferentiated proliferation of NPCs. To test this we compared marker expression on Matrigel versus FN1. Surprisingly, FN1 drastically reduces expression of the progenitor markers Cited1 and Six2, while strongly increasing Lef1 expression (Fig. 3A). For this reason we have excluded

FN1 as a candidate. Comparison of CITED1 expression in NPCs cultured on other ECMs revealed that nephronectin and Matrix 511 (a proprietary commercial ECM preparation based on laminin) had expressed Cited1, Six2, and Lef1 similarly to cells cultured on Matrigel (Fig. 3B-D). The XL313 antibody, which is directed against a cryptic collagen epitope reduced the expression of Lef1 significantly, implying that denatured collagen may be a determinant of NPC differentiation.

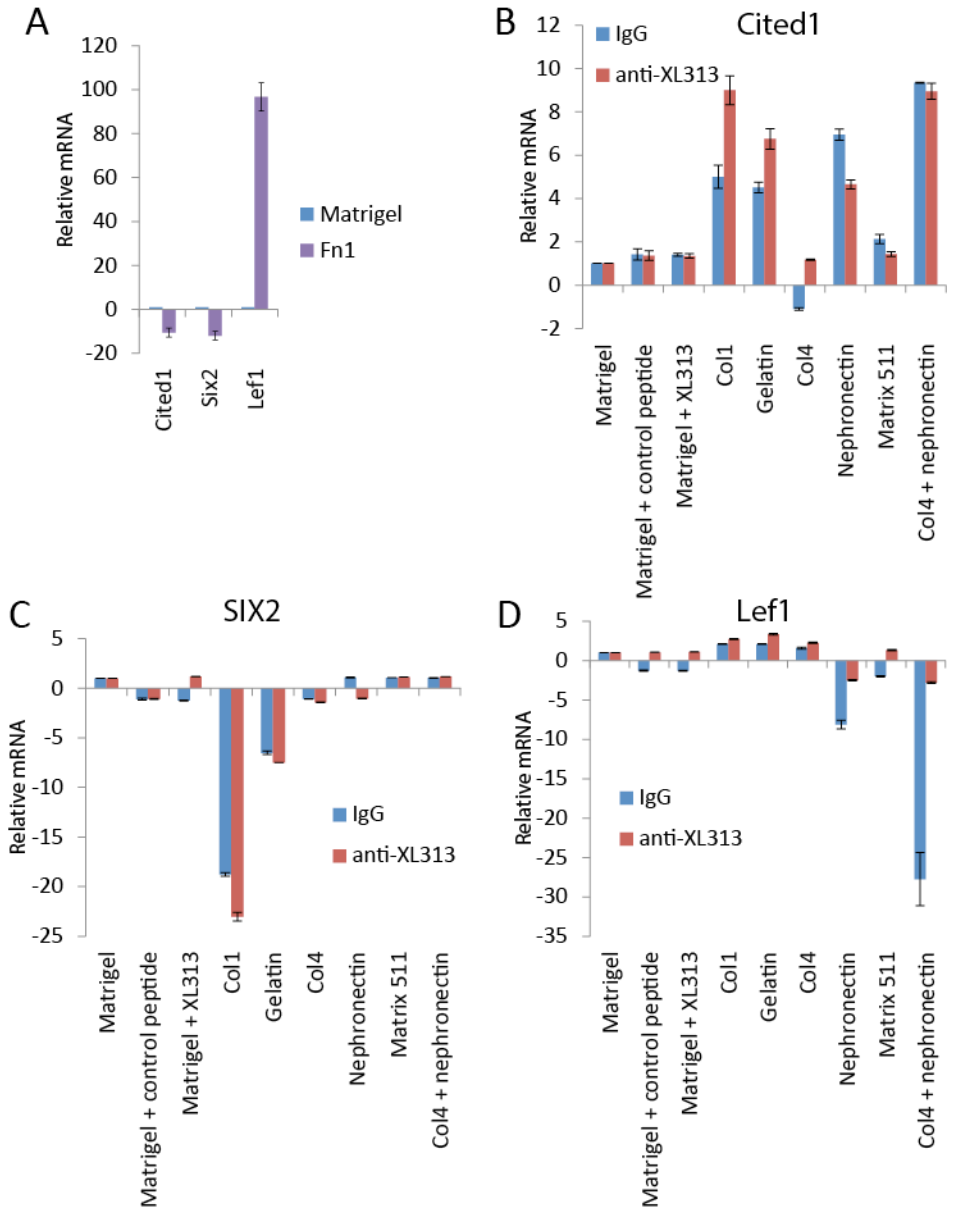

Figure 3. The effect of various ECMs on nephron progenitors grown in NPEM 2. (A-D) qPCR of progenitor markers from cells grown in monolayer with different ECM combinations and RGD blocking antibody XL313. Control peptide is the negative control for XL313 that lacks the RGD motif. XL313 and control were added to the medium at 25ug/ml. IgG represents the isotype matched antibody control.

In addition to reducing Lef1 expression, XL313 antibody increased cellular yield from NPC cultures, in many cases doubling the amount of RNA harvested from each culture (Fig. 4).

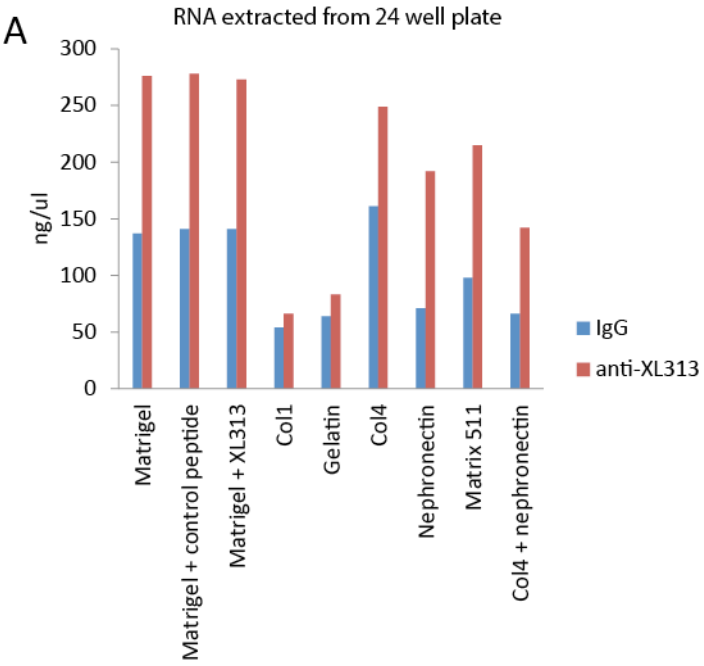

Figure 4. XL313 blocking antibody results in increased proliferation of nephron progenitors. Concentrations of RNA derived from samples shown in Figure 3 (B-D) are indicative of increased proliferation.

While the finding that nephronectin may substitute for Matrigel in undifferentiated NPC culture is scientifically valuable, it does not decrease the cost of NPC culture. Rather, it provides an alternate strategy for cell growth that should reduce experimental noise. XL313 is likewise a step forward scientifically that may inform future studies of the role of ECM in NPC differentiation.

NPEM 2 formulation
FGF2 150 ng/ml
BMP7 50 ng/ml
Y27632 10 μM
LDN193189 125 nM
CHIR99021 1 μM
IGF1 20 ng/ml
IGF2 2 ng/ml
All added to APEL medium