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Surface chemistry regulates valvular interstitial cell differentiation *in vitro*

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ABSTRACT

The primary driver for valvular calcification is the differentiation of valvular interstitial cells (VICs) into a diseased phenotype. However, the factors leading to the onset of osteoblastic-like VICs (obVICs) and resulting calcification are not fully understood. This study isolates the effect of substrate surface chemistry on *in vitro* VIC differentiation and calcified tissue formation. Using ω-functionalized alkanethiol self-assembled monolayers (SAMs) on gold [CH_3 (hydrophobic), OH (hydrophilic), COOH (COO^-, negative at physiological pH), and NH_2 (NH_3^+, positive at physiological pH)], we have demonstrated that surface chemistry modulates VIC phenotype and calcified tissue deposition independent of osteoblastic-inducing media additives. Over seven days VICs exhibited surface-dependent differences in cell proliferation ($COO^- = NH_3^+ > OH > CH_3$), morphology, and osteoblastic potential. Both $NH_3^+$and CH_3-terminated SAMs promoted calcified tissue formation while COO^--terminated SAMs showed no calcification. VICs on NH_3^+-SAMs exhibited the most osteoblastic phenotypic markers through robust nodule formation, up-regulated osteocalcin and α-smooth muscle actin expression, and adoption of a round/rhomboid morphology indicative of osteoblastic differentiation. With the slowest proliferation, VICs on CH_3-SAMs promoted calcified aggregate formation through cell detachment and increased cell death indicative of dystrophic calcification. Furthermore, induction of calcified tissue deposition on NH_3^+ and CH_3-SAMs was distinctly different than that of media induced osteoblastic VICs. These results demonstrate that substrate surface chemistry alters VIC behavior and plays an important role in calcified tissue formation. In addition, we have identified two novel methods of calcified VIC induction *in vitro*. Further study of these environments may yield new models for *in vitro* testing of therapeutics for calcified valve stenosis, although additional studies need to be conducted to correlate results to *in vivo* models.

Statement of Significance

Valvular interstitial cell (VIC) differentiation and aortic valve calcification is associated with increased risk of mortality and onset of other cardiovascular disorders. This research examines effects of *in vitro* substrate surface chemistry on VIC differentiation and has led to the identification of two materials-based initiation mechanisms of osteoblastic-like calcified tissue formation independent of soluble signaling methods. Such findings are important for their potential to study signaling cascades responsible for valvular heart disease initiation and progression as well providing *in vitro* disease models for drug development. We have also identified a VIC activating *in vitro* environment that does not exhibit confluence induced nodule formation with promise for the development of tissue regenerating scaffolds.
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1. Introduction

Valvular heart disease (VHD) is estimated to affect 2.5% of the US population with a disproportionate impact on an increasing elderly community [1,2]. Derived from infections, valve degeneration, or

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genetic disorders, VHD can manifest as regurgitation or stenosis of the valve. As a result of obstructed blood flow, valve stenosis generates transvavlular gradients that triggers compensatory ventricular hypertension, leading to increased risk for other cardiovascular disorders [1–3]. As such, VHD represents a significant health risk worldwide. Furthermore, there will continue to be rises in the prevalence of VHD as the elderly population grows due to advances in medical technology [1–5].

The leading cause of VHD is valve stenosis, which is characterized by valve thickening, increased protein deposition, and eventual calcification [3,4,6]. The primary driver for valvular calcification is the differentiation of valvular interstitial cells (VICs) into a disease inducing phenotype [6,7]. However, the factors leading to the onset of VIC differentiation and resulting calcification are not fully understood and a more complete characterization of VIC differentiation and phenotypic change is required before treatment of valve disease can be realized.

As the predominant cell type within the valve, VICs are responsible for valve formation, remodeling, and tissue homeostasis. VICs are a heterogeneous population that undergo phenotypic change regulated by environmental factors. VICs have been shown to alter phenotype in response to soluble factors [8–11], substrate stiffness [6,8,12–15], and surrounding extracellular matrix proteins [8,10,11,16–21]. Although phenotypic change is a dynamic event, literature tends to compartmentalize VIC function in order to clarify and separate the underlying causes [22].

VICs are commonly characterized by their quiescent (qVIC), activated (aVIC), and disease phenotypes. In a normal adult valve, qVICs are thought to preserve physiological structure and function while maintaining a low level of matrix synthesis and degradation inhibiting angiogenesis [22]. Following injury or abnormal hemodynamic/mechanical stress, VICs become activated (aVICs, myofibroblastic) and are associated with increased extracellular matrix secretion and expression of various biological markers including α-smooth muscle actin (α-SMA), matrix metalloproteinases, and transforming growth factor-β [22,23]. Activated VICs exhibit increased contraction, prominent stress fiber formation, increased proliferation, and migration [22]. *In vitro*, aVICs display an elongated morphology and form orthogonal patterns of overgrowth resembling hills and valleys [6]. Upon completion of remodeling or wound healing, most aVICs are eliminated by apoptosis or reversion to qVICs [24].

When VIC dysregulation or abnormal extracellular matrix production occurs, conversion to diseased phenotypes results in pathological fibrosis and calcification of the valve [10,24]. The mechanisms of VICs tissue calcification have been intensely study and currently two processes have been proposed [5,25–27]. Dystrophic calcification is a passive degenerative process characterized by early cell injury and deposition of calcium associated with tissue damage and necrotic cells [10,25,26]. Alternatively, osteogenic calcification (ossification) is an active process involving bone and cartilage development, marked by the expression of various osteogenic markers [7,25,26,28]. *In vitro*, VICs undergoing osteogenesis, osteoblastic-like VICs (obVICs), are characterized by a round/rhomboid morphology followed by the formation of three-dimensional calcified nodules. It is important to note, that these two processes are not mutually exclusive as a common activator of tissue calcification, TGF-β, has been shown to lead to cellular apoptosis as well as nodule formation [10]. However, different processes may arise due to variations in signaling in the cellular microenvironment. Forced obVIC differentiation *in vitro* is commonly achieved by adding soluble signaling factors (β-glycerophosphate, ascorbic acid, and dexamethasone) to media. However, spontaneous *in vitro* nodule formation in media lacking osteoblastic inducing additives [8], as well as calcification of decellularized biological valves *in vivo*, suggests calcification may be mediated by additional physical cues [29].

To understand how surface chemistry correlates to VIC behavior and initiation of valvular disease, we used alkanethiol self-assembled monolayers (SAMs) of ω-functionalized alkanethiolates on gold as model substrates with uniform chemistry. The chemisorption of thiols to gold and the hydrophobic interactions of alkane chains results in stable, ordered, and well-packed monolayers (Fig. 1) [30]. As such, SAMs offer well-defined models for systematically investigating phenotypic change directed by surface chemistry. Four physiologically relevant functional groups were used to assess the effects of substrate hydrophobicity and charge on VIC phenotypic behavior. Due to the negatively charged environment present during valvulogenesis through increased hyaluronic acid expression, it was hypothesized that anionic functional groups would result in VIC activation and tissue production [31,32].

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were purchased from Sigma–Aldrich chemicals unless otherwise noted. All cell culture media and reagents were purchased from Thermo Fisher Scientific, unless otherwise noted.

2.2. Fabrication of self assembled monolayers

Gold-coated round 15 mm glass cover slips (26021, Ted Pella Inc.) were used as substrates for self-assembled monolayer (SAMs) formation. Coverslips were etched for 30 min prior to gold deposition in Piranha solution [70% (v/v) concentrated H_2SO_4, 30% industrial grade H_2O_2 (KMG Chemicals)] rinsed with diH_2O, and blown dry with N_2. Gold coating was conducted by sequential electro-evaporation of optically transparent films of chromium, adhesion layer (2 nm; High Vacuum Evaporator Systems), followed by gold (30 nm, 99.99% purity; Plasmaterials). Metal deposition was accomplished at 2 nm/s using a Thermionics VE-90 vacuum Evaporation System (TLI Enterprises) with chamber pressures at or below 1×10^{-5} Torr. Freshly prepared gold substrates were immersed in 1 mM ethanolic alkanethiol solutions [1-dodecanthiol (47-136-4);

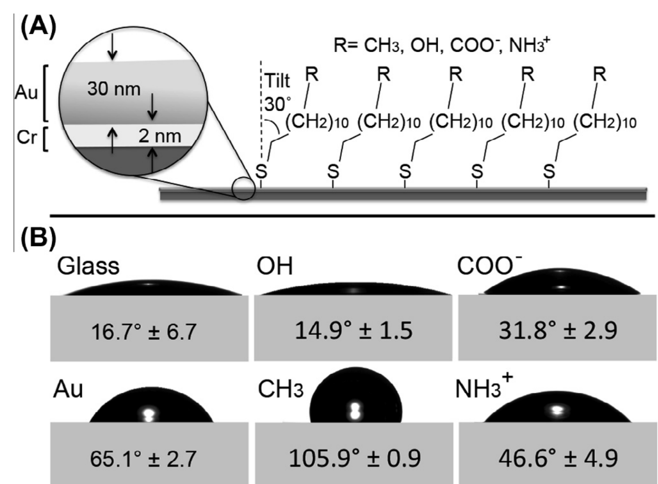

Fig. 1. (A) Schematic of alkanethiol self-assembled monolayer (SAM) on gold (Au, 30 nm) coated glass with chromium (Cr, 2 nm) adhesion layer. (B) Air–water–surface contact angles determined using the sessile drop goniometry (ultrapure H_2O in air).

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